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# Experimental Demonstration of Tongue Muscle Origin in Chick Embryos

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WITH ONE PLATE

SINCE in all classes of vertebrates the tongue muscles are innervated by nerve XII, a segmental nerve of the occipital region, it is usually argued on this criterion alone that they originate from occipital myotome tissue. Descriptive evidence in support of this generalization is, however, far from adequate. The most complete accounts that exist refer to one amphibian and two reptile species. In the amphibian *Necturus*, Platt (1897) observed that ventral outgrowths of the 3rd and 4th occipital myotomes became tongue muscles, and Edgeworth (1935) has described the development of tongue muscles in the reptiles *Sphenodon* and *Lacerta*, from ventral parts of two occipital and two cervical myotomes, all innervated by nerve XII. In avian and mammalian embryos, however, early muscle rudiments are extremely difficult to recognize with any certainty histologically. Although Butcher (1929) and Bates (1948) have observed ventral outgrowths of occipital myotomes in rat and cat embryos, and Bates claimed that these are recognizable as tongue muscle primordia in later stages, both these authors drew their conclusions from relatively few fixed specimens, and in Bates's photographs the rudiments are not nearly as distinct as his diagrams suggest they are. The same criticism applies to Hunter's description (1935) of occipital myotome rudiments that apparently develop into tongue muscles in the chick. The only other detailed description of tongue muscle development in birds is that of Kallius (1905) who dealt with advanced stages when the muscle primordia are already *in situ*.

In the work to be described here, an experimental approach to the problem has been adopted, and occipital somites of early chick embryos have been marked with carbon particles in order to trace their later fate. Forty-five-hour embryos (stages 10–12, Hamburger & Hamilton, 1951) were operated on *in ovo* via a window in the shell. Carbon particles, moistened slightly with Pannett-Compton saline, were pushed into one of the first three post-otic somites on the

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right-hand side of the embryo with a fine tungsten needle. In all cases, jabs with the needle were continued until it was quite certain that carbon had firmly penetrated the somite; then excess carbon particles lying on the vitelline membrane were removed with a micropipette. After replacing the shell 'window' the embryo was reincubated for a further 3–4 days, then fixed in Bouin's fluid, imbedded by Peterfi's celloidin-paraffin technique, sectioned usually sagittally, and stained with Weigert's haematoxylin and eosin or with Delafield's haematoxylin and Congo red.

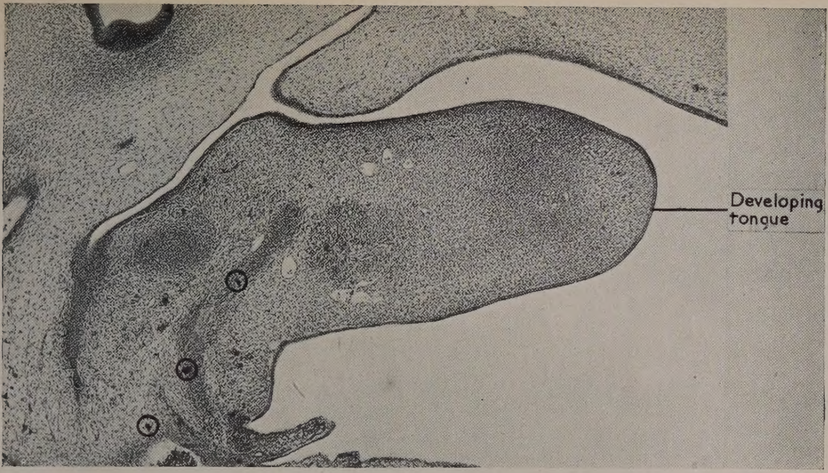
Mortality was high in these experiments: out of 98 operated embryos only 25 survived long enough to be suitable for histological examination, and only 6 of these were actually alive at the end of the experiment. The chief causes of death seemed to be adherence of the vitelline membrane to the shell window, or splitting of the vitelline membrane when the original hole made with the needle had been somewhat large. There was also in some embryos an extensive vasodilatation, which may have been due to blockage of the blood-vessels with carbon particles, since particles were sometimes seen to escape into the vitelline arteries at the time of operation.

However, despite the low yield, the results were very consistent. In 21 out of the 25 embryos examined histologically, carbon had reached the tongue region on the right-hand side and was specifically localized here, hardly any occurring in other parts of the pharynx. In the 4 embryos where this was not so, death had occurred at an early stage (3 days) and in 2 of them carbon was scattered throughout the somewhat macerated tissues, making it impossible to say that it was localized in any particular region. The 2 others which had also met an early death showed carbon still at the ventral borders of the 2nd and 3rd post-otic somites.

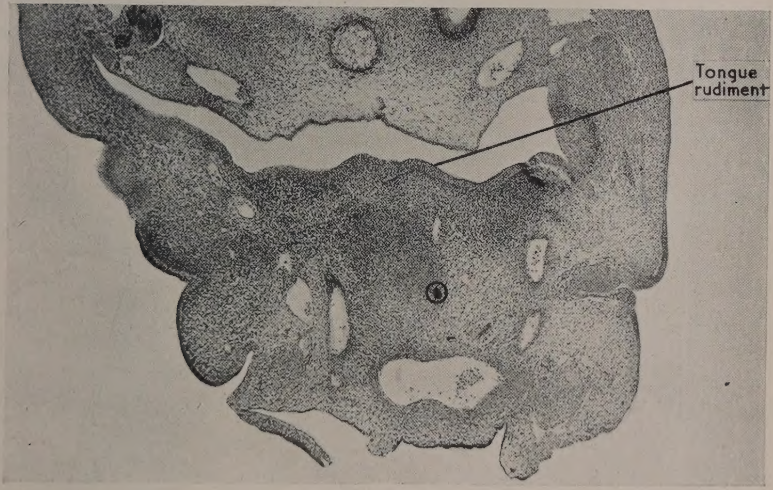
The 6 embryos that were living at the time of fixation (stages 25–28, Hamburger & Hamilton) had well-developed tongue rudiments with muscle already evident, and in 5 of these carbon was found in, and adjacent to, the geniohyoid muscle on the right-hand side (Plate, figs. A, B). There were 4 more embryos (stages 20–22) where the tongue rudiment had as yet no clear muscle, and 3 of these showed carbon particles in the tongue mesoderm; the 4th had carbon in near-by mesoderm of the mandibular arch.

There remain to be considered 11 embryos which had died too early to show a tongue rudiment. In 8 of these carbon had reached the mandibular arch mesoderm of the right side, in 2 others carbon lay near the base of the right mandibular arch, and in the remaining one there were scattered carbon particles in the mesoderm of the pharyngeal floor.

The conclusion may be drawn from these experiments that in chick embryos cells from the occipital somite region do indeed migrate ventralwards to the lower jaw and participate in the formation of tongue muscles. Hence the innervation of these muscles by nerve XII is understandable, and the generalization on which previous statements about tongue muscle origin have been based—



A



B

E. M. DEUCHAR

namely, that when muscles migrate during development they carry their nerve-supply with them—seems fully justified for this particular case.

## SUMMARY

Carbon marks placed in the occipital somites of 45-hour chick embryos have been observed in mandibular arch tissue at later stages, and within the tongue muscles when these have developed. Thus the occipital origin of tongue muscles in the chick seems clearly established.

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## EXPLANATION OF PLATE

FIG. A. Carbon particles (ringed) in and near the geniohyoid muscle. Sagittal section. × 73.

FIG. B. Transverse section through mouth region of a 5½-day chick embryo. Carbon mass (ringed) in geniohyoid muscle. × 83.

*(Manuscript received 20 : iii : 58)*

# The Melanoblast Reservoir Available to a Feather Papilla

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WITH TWO PLATES

## INTRODUCTION

IT has been established beyond doubt that the melanin pigments of feathers are produced by cells which, like other vertebrate melanin-producing cells, are ultimately derived from the embryonic neural crest (Rawles, 1948). The migratory process whereby the skin receives prospective pigment cells from the neural crest occurs in early embryonic life; in the chick embryo it is completed by 90–100 hours of incubation (Watterson, 1942). The later stages of the migration, whereby cells of this lineage come to lie in intimate contact with the epidermal cells of a developing feather, are more controversial. The definitive pigment cells (melanocytes) are lost into feathers, so that there must exist some reservoir of melanoblasts from which the melanocytes of future feathers are derived.[3] It is with the nature and location of this reservoir that we are here concerned. Before describing our experiments it will be advisable to discuss some of the previous literature.

Foulkes (1943) has put forward the view that the reservoir is located outside the feather follicle. His own experiments do not support this view unequivocally;

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[3] The term 'melanoblast' denotes an immature pigment cell, and the criterion of maturity generally adopted is the production of pigment; a melanocyte produces and contains melanin, and a melanoblast does not (Gordon, 1953). In this paper we shall use the term melanoblast to denote a cell with substantial powers of division and regeneration: a cell potentially capable of producing descendants which will contribute pigment to a *future* feather. This is purely a matter of terminological convenience; we do not wish to imply that the loss of regenerative powers necessarily coincides with the start of melanin production, nor even that the loss occurs suddenly and in one step. Mitosis can occur in pigment cells which already contain pigment (Watterson, 1942), and there may exist in a developing feather cells capable of contributing melanocytes to more proximal parts of the same feather, but not to a subsequent feather. Such cells, if they exist, we would on our present usage call melanocytes, not melanoblasts.

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indeed, they show that the papilla of a resting follicle does (sometimes, at least) contain melanoblasts. Nevertheless, Foulkes held that the melanoblast content of the resting papilla, or of the papilla left behind when a partly grown feather is plucked, is too meagre to make a significant contribution to subsequent feathers, whose melanocytes he believed to be derived from melanoblasts drawn into the papilla at the start of growth of each new feather from the dermal tissues surrounding the base of the follicle..

In the case of the mammalian hair follicle, an intra-follicular reservoir of melanoblasts has been postulated by Rawles (1948, 1955) among others. According to this view the hair follicle, once fully developed, is a closed system, and the melanocytes of all hairs produced by the follicle are derived solely from the initial stock of melanoblasts incorporated into the papilla during early development. Rawles (1948) appears to hold a similar view in relation to feather follicles, quoting Watterson (1942) to this effect. However, all that Watterson (1942) claimed to have shown was that melanoblasts reach the wing-tips of Barred Plymouth Rock embryos too late (after 91 hours) to be incorporated into the follicles which subsequently develop there, thus explaining the fact that the down feathers of the wing-tips are unpigmented. Since these same follicles later produce pigmented definitive feathers, any closure of the follicles to entry of melanoblasts can be no more than temporary. There is, moreover, no evidence at all that the whiteness of the wing-tip down feathers is due to absence of melanoblasts; it is curious that Watterson does not even mention the far more characteristic white occipital patch in the down of Barred Plymouth Rock chicks —a location where late arrival of melanoblasts is particularly unlikely. Those regional differences in pigmentation in fowl plumage which have been adequately analysed (e.g. in the work of Willier & Rawles (1940), Rawles (1944), and Cohen (1957), and in the results to be described in this paper) have been shown to be due, not to regional differences in the quantity or inherent quality of melanoblasts, but to a response of the melanoblasts to local conditions within the follicle.

That the unconditional barrier to entry of melanoblasts postulated by Rawles and others in the hair follicle does not exist in the feather follicle is shown decisively by the development of host-pigmented plumage in Rawles's (1944) grafts of melanoblast-free skin onto newly hatched chicks. This proves that melanoblasts will migrate into skin and feather follicles which have been experimentally deprived of their own normal complement of melanoblasts. It remains unproven that a *normal* feather follicle ever lacks at any stage a complement of melanoblasts, so that one cannot infer that the melanocytes of feathers are normally derived from an extra-follicular reservoir.

Patches of donor pigmentation produced in the plumage of chicks by implanting tissue containing melanoblasts into the wing-buds of 3-day embryos are always replaced by host pigmentation within a few weeks or months, whatever the respective colours of donor and host (Willier & Rawles, 1940). The replacement of donor by host pigmentation occurs quite sharply, often in mid-growth of a feather. The cause of this 'dying-out' of donor melanoblasts is obscure; the early age of implantation should provide ideal conditions for the development of acquired tolerance (Billingham, Brent, & Medawar, 1956) so that an immunological cause seems unlikely. Nor do limited developmental potentialities of the donor melanoblasts seem to be responsible, since replacement occurs even when the implanted material consists of embryonic neural crest—that is to say, presumptive rather than definitive melanoblasts (Dorris, 1939). Until the cause of this dying-out is understood, it cannot be taken as conclusive evidence that a similar replacement of one set of melanoblasts by another occurs in *normal* feathers.

The phenomenon of 'ticking' or 'flecking' is again relevant but inconclusive. Patches of various shapes and sizes with the recessive type of pigmentation occur copiously in the plumage of fowls heterozygous for certain dominant or semidominant colour and pattern genes (*B*, *Bl*, *I*, and *S*). When successive generations of feathers from the same follicle are compared these 'ticks' show little or no tendency to recur in the same follicles, except where a single tick is large enough to cover several adjacent follicles (Montalenti, 1934; Juhn, 1933, and unpublished observations by Cock). For the sex-linked *B* and *S* loci there is strong evidence (Cock, 1953) that the melanocytes forming the ticks have lost part or all of the chromosome bearing the dominant gene. On this explanation the melanocytes (and, in the case of the larger ticks, the melanoblasts too) are *ex hypothesi* abnormal, and this abnormality may be reflected in a competitive disadvantage. Thus the frequent replacement of a ticked feather by a normally pigmented one does not necessarily give a valid indication of the normal rate of turnover of melanoblasts. Moreover, there is no direct evidence that the replacing melanoblasts come from outside the follicle; it may be that the papilla contains a relatively large reservoir of melanoblasts, only some of which take part in the pigmentation of any one feather. This possibility, as will be seen, receives some support from our present work.

Danforth (1929, 1939) found occasional mosaic feathers at the edges of tolerated skin homografts. Successive feathers from the same follicle were usually, but not always, of similar type. There is again uncertainty as to the immediate source of the melanoblasts; it is likely that some or all of the mosaic feathers arose from papillae cut through or otherwise damaged in the grafting operation.

Lillie & Wang (1941, 1943, 1944) and Wang (1943) have analysed the morphogenetic functions of the feather papilla by means of an extensive series of autotransplants of papillae and parts of papillae. They have shown that the dermal core of the papilla acts as an inductor of follicle formation, and determines the orientation of the feather, whereas regional differences in feather structure are determined entirely by the regional origin of the epidermal covering of the papilla. Wang (1944) has carried out homotransplants of papillae between differently coloured breeds. The pigmentation of the resultant feathers was in all cases of host type, a result which Wang interpreted as supporting Foulkes's (1943) hypothesis of an extra-follicular reservoir of melanoblasts. It is abundantly clear that, as might be expected, the hosts developed immunity against Wang's homotransplants; the proportion of complete failures and of seriously defective (tuft-like) feathers was much higher than in comparable autotransplants. Moreover, not only the melanoblasts, but also the epidermis of the feathers was exclusively of host origin. The dermal cores of some of the transplants may have induced the formation of new papillae from host tissue, but it seems likely that the transplants themselves were entirely destroyed by the immune reaction of the host. Critical evidence as to the location of the melanoblast reservoir can be obtained by homotransplants of papillae only if adequate precautions are taken to avoid the occurrence of transplantation immunity. We have now carried out homotransplants with such precautions, and our results are quite different from those obtained by Wang.

## MATERIALS AND METHODS

There are two ways in which transplantation immunity may be avoided. The first makes use of the fact that grafts from an isogenic inbred line are acceptable by an $F_1$ cross with any other line. The lack of sufficiently inbred lines of suitable colours has so far prevented the use of this method. Preliminary test grafts of wattle skin were made at Cambridge from five inbred Rhode Island Red hens (eight generations of full-sib mating) onto $F_1$'s with an inbred line of Cuckoo Leghorns, but in each case the homograft was rapidly destroyed by the host.

The second method makes use of 'acquired tolerance' (Billingham, Brent, & Medawar, 1956). Two birds were available at Cambridge which, as newly hatched chicks, had received homografts of back (saddle) skin (Danforth & Foster, 1929) by the technique of Cannon & Longmire (1952). These were survivors from a batch of 34 chicks, from matings segregating for several colour genes, grafted in August 1954. The grafts on most of the other chicks had sooner or later succumbed to homograft reactions, but these two were permanently tolerated. Both hosts (♂ 3774/4 and ♀ 3780/4) were of rather nondescript 'black-red' pigmentation pattern (i.e. very roughly similar to Brown Leghorns), each bearing a white-feathered graft some 6 × 6 cm. orientated transversely on the back (Plate 1, fig. 1). The donor chicks had both been heterozygous for dominant white (*I*) and showed occasional blue-grey ticks in the plumage (Plate 1, fig. 4).

Hamilton (1940) has shown that the growing feathers of White Leghorns (homozygous for *I*) do contain melanocytes. At the very base of the growing feather (the collar) melanocytes containing pigment granules are present, but in the region of barb-formation (where, in the feathers of a coloured breed, the rate of pigment deposition is at its maximum) the melanocytes become degenerate and die. The homografts on our two birds are therefore to be regarded as possessing melanoblasts which do produce melanocytes. These melanocytes are capable of 'occupying' a feather to the exclusion of other melanocytes (see Rawles, 1944), but do not produce sufficient pigment to give any appreciable colour to the feather. Further support for this view is available from genetical evidence which shows that *I* is an inhibitor, not of melanocytes, but of certain types of pigment (Cock, 1956). The main effect of *I*, at least when heterozygous, is to cause those parts of the plumage which would otherwise be black or grey to be white; red, brown, or yellow parts of the plumage are relatively unaffected, although their colour may be diluted. Only when other genes (such as *E*) have eliminated all the red, brown, or yellow pigment, or replaced it by black, does the addition of *I* yield an entirely white phenotype.

The occasional ticks in the homograft plumage depend on the fact that the donors were only heterozygous for *I*; they may have been produced by melanocytes which had lost the *I* gene (see Introduction). Whether or not this is true, these ticks need cause no confusion in interpreting our results. Because of other genes (*B*, *Bl*, and *E*) carried by the donors, the pigmentation of the ticks (blue-grey, sometimes with traces of diffuse and irregular transverse barring, and quite devoid of red, brown, or yellow pigment) is quite distinct from the black-red pigmentation pattern of the hosts.

Transplants of feather papillae between the host and its own homograft were carried out on these two birds at Hull in October 1956, and the birds were then returned to Cambridge. The method was to interchange whole papillae between follicles of the homograft and of a host tract of distinctive feather structure. The operative technique was essentially that described by Lillie & Wang (1941). All papillae exchanged were active ones, from follicles containing pin-feathers. Feathers subsequently growing from the transplanted papillae could be identified by their structure (that of the donor tract) and in many cases by their abnormal orientation. Tattoo marks in the skin provided an additional aid in identifying follicles. Examinations were made, and notes taken, of the structure, colour, orientation, and stage of growth of feathers, at intervals of about 2 weeks.

It was anticipated that the source from which the melanocytes of a feather were derived might perhaps depend on whether the previous feather from the same papilla had been plucked as a pin-feather, or had been allowed to complete its growth (Foulkes, 1943). Some post-operative feathers were therefore plucked before completing growth; others were left fully grown for varying periods before being plucked.

In order to stimulate inactive follicles, ♀ 3780/4 was given an intramuscular injection of 40 mg. progesterone on 7 February 1957, and a further course of nine subcutaneous injections into the leg, each of 20 mg. progesterone, from 21 May to 1 June 1957. Progesterone stimulates feather growth without affecting structure or pigmentation (Shaffner, 1954). The Poultry Genetics Unit at Cambridge ceased to exist in October 1957. ♂ 3774/4 was then killed, as it seemed unlikely to yield any further information of value. ♀ 3780/4 was transferred to Hull, where it is now under the care and observation of Professor P. G. 'Espinasse.

## RESULTS

### (*a*) ♂ *3774/4*

It proved very difficult to maintain a satisfactory state of anaesthesia in this bird, and for this reason only three papillae from the homograft were implanted into emptied follicles on the breast. Six papillae had been removed from the breast; three of these were implanted into the emptied follicles on the homograft, the other three being implanted into incisions in the skin of the homograft where they would be expected (Lillie & Wang, 1941) to induce the formation of new follicles. Only one feather (Plate 1, fig. 6) has been identified as growing from a transplanted papilla, and the interpretation of even this is rather dubious. The haste with which the operations had to be carried out is probably partly responsible for the almost complete failure of the transplants on this bird. It is also possible that a very mild form of transplantation immunity played some part in the failure. The homograft on this bird (unlike that on ♀ 3780/4, which had been completely healthy and vigorous throughout) had shown signs—a transient inflammation and a delay in the start of feather growth—of a very mild homograft reaction during the first few weeks after the original grafting operation. It is conceivable that a very weak state of immunity, sufficient to interfere with the process of healing in, but not to affect well-established homograft tissue, had persisted into adult life.

### (*b*) ♀ *3780/4*

The papillae were removed from ten follicles on the neck, and from ten follicles on the homograft. The ten papillae from the neck were implanted into the emptied follicles on the homograft, and vice versa. The typical structure and pigmentation of feathers of these tracts is illustrated in Plate 1, figs. 2, 3, 4. Eleven of the transplanted papillae have since grown one or more feathers each: the results are summarized in Table 1, and some of the feathers are shown in Plate 1, fig. 5, and Plate 2, fig. 7. The remaining nine papillae presumably failed to survive transplantation. The rather frequent occurrence of dwarfed and otherwise defective feathers is presumably due to mechanical damage sustained by the papillae during transplantation.

It will be seen that all six neck papillae in the homograft have so far produced only black-red feathers. Structure, where not obscured by defects, is of neck type, as is pigmentation pattern (feathers E 1 and E 2—Plate 1, fig. 5).

Of the five successful transplants from the homograft into the neck, one (U) has so far produced only white feathers. Each of the other four has produced both white and black-red, either in successive feathers, or in different parts of the same feather. The first four feathers produced by O are entirely white; the fifth is mosaic, predominantly black-red with a small white patch. Q has grown two white feathers, followed by a black-red feather. P has grown five feathers;

## TABLE 1

*♀ 3780/4: histories of transplanted papillae and of a spontaneously mosaic follicle on the homograft*

Feathers grown from the same follicle are designated by the same capital letter, and are numbered in order of growth. The arrow in the first column indicates the approximate orientation of the feathers in relation to the normal orientation (↑) in the recipient tract. The three dates in the second column are the dates when growth of the feather began and was completed (both subject to an error of about ±5 days) and the date when the feather was plucked, or fell out of the follicle. A dash in place of the second date indicates that the feather was plucked or lost before completing growth. A + indicates that the feather had not begun growth by 8 Oct. 1957. The bird was next examined on 13 Feb. 1958; all feathers except R 5 were then fully grown. The pigmentation type of ♀ 3780/4 herself (including the neck) is black-red; the homograft is white-feathered.

| *Follicle* | *Feather* | | *Structural type* | *Pigmentation* | *Other comments* |
|---|---|---|---|---|---|
| *Papillae from neck transplanted into emptied follicles on homograft* (*saddle*) | | | | | |
| A | A 1 | 10.11.56<br>—<br>17.12.56 | ?<br>(Dwarfed and defective) | Black-red | Lost before plucking |
| B | B 1 | 10.11.56<br>—<br>17.12.56 | ?<br>(Dwarfed and defective) | Black-red | Lost before plucking |
| C | C 1 | 10.11.56<br>—<br>17.12.56 | Neck?<br>(Dwarfed and defective) | Black-red; near neck type | — |
| | C 2 | 15. 1.57<br>—<br>7. 2.57 | ?<br>(Dwarfed and defective) | Black-red | — |
| D | D 1 | 10.11.56<br>—<br>17.12.56 | ?<br>(Dwarfed and defective) | Black-red | Lost before plucking |
| E ↗ | E 1 | 10.11.56<br>20.12.56<br>10. 1.57 | Neck | Black-red; neck type | — |
| | E 2 | +<br>+<br>13. 2.58 | Neck | Black-red; neck type | — |
| F ↘ | F 1 | 10.11.56<br>25.12.56<br>10. 1.57 | ?<br>(Dwarfed and defective) | Black-red | — |
| | F 2 | 10. 6.57<br>20. 7.57<br>12. 8.57 | Neck<br>(Dwarfed) | Black-red; neck type | — |
| | F 3 | +<br>+<br>13. 2.58 | ?<br>(Dwarfed and defective) | Black-red | — |

TABLE 1 (*cont.*)

*♀ 3780/4: histories of transplanted papillae and of a spontaneously mosaic follicle on the homograft*

| *Follicle* | *Feather* | | *Structural type* | *Pigmentation* | *Other comments* |
|---|---|---|---|---|---|
| *Papillae from homograft (saddle) transplanted into emptied follicles on neck* | | | | | |
| O ↙ | O 1 | 5.11.56<br>20.12.56<br>10. 1.57 | Saddle | White | — |
| | O 2 | 15. 1.57<br>—<br>7. 2.57 | Saddle | White | Vane broke off before plucking |
| | O 3 | 5. 3.57<br>—<br>27. 3.57 | Saddle | White | — |
| | O 4 | 10. 6.57<br>20. 7.57<br>12. 8.57 | Saddle | White | — |
| | O 5 | +<br>+<br>13. 2.58 | Saddle | Mosaic; white patch on right margin of vane; remainder black-red (saddle-type) | — |
| P↑ | P 1 | 10.11.56<br>—<br>20.11.56 | ?<br>(Defective) | Black-red | Lost before plucking |
| | P 2 | 25.11.56<br>15. 1.57<br>7. 2.57 | Saddle | Black-red; saddle-type | — |
| | P 3 | 15. 2.57<br>—<br>27. 3.57 | Saddle | Mosaic; white patch at tip; remainder black-red (saddle-type) | — |
| | P 4 | 10. 6.57<br>20. 7.57<br>12. 8.57 | Saddle | Mosaic; white patches at tip and on left margin of vane; remainder black-red (saddle-type) | — |
| | P 5 | +<br>+<br>13. 2.58 | Saddle | Mosaic; similar to P 4 but both white patches much smaller | — |
| Q ← | Q 1 | 15.11.56<br>—<br>17.12.56 | Saddle | White | — |
| | Q 2 | 29.12.56<br>21. 2.57<br>13. 3.57 | Saddle | White | — |
| | Q 3 | 10. 6.57<br>—<br>12. 8.57 | ?<br>(Dwarfed and defective) | Black-red | — |

TABLE 1 (*cont.*)

♀ *3780/4: histories of transplanted papillae and of a spontaneously mosaic follicle on the homograft*

| *Follicle* | *Feather* | | *Structural type* | *Pigmentation* | *Other comments* |
|---|---|---|---|---|---|
| | | | *Papillae from homograft (saddle) transplanted into emptied follicles on neck* | | |
| R↓ | R 1 | 15.11.56<br>—<br>17.12.56 | Saddle | Black-red; saddle-type | — |
| | R 2 | 1. 1.57<br>—<br>16. 2.57 | Saddle | White | Lost before plucking |
| | R 3 | 21. 2.57<br>—<br>27. 3.57 | Saddle | White | — |
| | R 4 | 5. 6.57<br>20. 7.57<br>12. 8.57 | Saddle | White | — |
| | R 5 | +<br>—<br>13. 2.58 | ?<br>(Defective; partially twinned) | Black-red | — |
| U↓ | U 1 | 5. 1.57<br>—<br>7. 2.57 | Saddle | White | — |
| | U 2 | 15. 2.57<br>—<br>27. 3.57 | Saddle | White | — |
| | | | *Unoperated intact follicle on homograft (saddle)* | | |
| G↑ | G 1 | †<br>†<br>17.12.56 | Saddle | Mosaic; black-red (saddle-type) central stripe; white edges | — |
| | G 2 | 10. 6.57<br>20. 7.57<br>12. 8.57 | Saddle | Mosaic; similar to G 1 | — |
| | G 3 | +<br>+<br>13. 2.58 | Saddle | Mosaic; left side is similar to G 1 and G 2, but almost the whole of the right side is black-red | — |

† G 1 was already fully grown when transplants were made on 19 Oct. 1956.

the two earliest are both entirely black-red; each of the other three is a mosaic, predominantly black-red with one or two small white patches. These white patches occur in similar positions in the three feathers. R has grown five feathers in the remarkable sequence: black-red, white, white, white, black-red. With the exception of a few feathers of defective structure, all the feathers grown from transplanted homograft papillae are typical saddle (back) feathers in shape and structure. The pigmentation pattern in the black-red feathers, and in the black-red parts of mosaic feathers, corresponds (with one qualification, to be noted later, in the case of the mosaic feathers) to that of the saddle feathers of the host. Since the black-red melanocytes in these feathers must have been derived from melanoblasts from the surrounding neck tissue, this is an added confirmation of the point mentioned in the Introduction, that regional differences in pigmentation pattern are not due to inherent differences between the melanoblasts of different regions of the skin.

One spontaneous mosaic feather (G 1—see Table 1, and Plate 2, fig. 9) was already present on the homograft when the transplants were carried out. The two feathers which have since grown from the same follicle as G 1 are also mosaic, with the pigmented (black-red) area occupying roughly the same parts of the feather. The pigmented parts of these three feathers—especially the pale brown rachis stripes—are quite distinct in appearance from the occasional ticks on normal homograft feathers (see above and Plate 1, fig. 4). G 1–3 differ from the spontaneous mosaics described by Danforth (1929, 1939) in that the follicle involved is *not* at the extreme edge of the homograft. It is about 1 cm. in from the edge, and is surrounded on all sides by follicles which have produced only normal white feathers.

It should be emphasized that in all seven mosaic feathers (G 1–3, O 5, P 3–5) the boundary between white and pigmented parts, although in places very irregular, is quite definite. There is no trace of any gradual dilution of pigment as one passes from coloured to white. On the contrary, the pigmentation near the boundary is abnormally intense. In every case the white area in the vane is outlined by a band of solid black, of rather irregular width (see Plate 2, fig. 8), contrasting with the normal brown-and-black mottled pattern of the vane of a saddle feather. Microscopic examination emphasizes the abnormal nature of the pigmentation in these black marginal bands. In the vane of the normal saddle feather the barbs are brown-pigmented throughout; the black mottling is due solely to areas of black barbules. In these marginal bands the barbs as well as the barbules are intensely black.

## DISCUSSION

These results seem to have the following implications:

1. The papilla of a feather follicle contains a reservoir of melanoblasts which may be sufficient to pigment at least four successive feathers (follicle O). It is

noteworthy that every one of the successful transplants has given evidence that some, at least, of the native melanoblasts have survived transplantation.

(2) Not all the melanoblasts within the papilla necessarily contribute melanocytes to any one feather. The inactive melanoblasts may, however, contribute some (follicle P) or all (follicle R) of the melanocytes utilized by subsequent feathers.

(3) The reservoir within the papilla may be augmented or replaced from time to time by melanoblasts migrating in from outside the papilla. The results so far do not show how frequently this occurs, nor whether it is influenced by factors such as prolonged inactivity of the follicle. Nor is there any compelling reason to suppose that this is a necessary event, without which the reservoir within the papilla would eventually be exhausted.

Some qualifications need to be added to these conclusions. The use of dominant white as a marker gene, which was dictated by the material available, introduces two difficulties. Firstly, it could be objected that some or all of the white in feathers from transplanted papillae is due, not to dominant white melanocytes, but to genetically black-red melanocytes which are malfunctioning, or to a complete absence of melanocytes. Sporadic and irregular whitening is not uncommon in the plumage of some coloured fowls, but it has never been observed in the normal plumage of ♀ 3780/4. This objection is probably not valid, therefore, in this case.

Secondly, it is possible that dominant white melanoblasts are in some way lacking in 'vigour', thus introducing an element of competition which would not be present in a normal papilla. Some such deficiency on the part of dominant white melanoblasts is suggested by their behaviour in tissue culture (Hamilton, 1940), and by the fact that, in the production of donor-coloured patches by implanting melanoblasts into three-day limb buds, the combination of coloured donor and dominant white host gives much better results than the reciprocal combination (Willier & Rawles, 1940; Dorris, 1939). The fact that none of our transplants into the homograft has so far given evidence of the entry of dominant white melanoblasts may be attributable to competitive disadvantage of dominant white melanoblasts, but fewer feathers have been grown from these transplants than from the reciprocal combination, and other factors, such as tract differences, may influence the readiness with which melanoblasts enter a papilla. Any competitive disadvantage of dominant white melanoblasts cannot be an overwhelming one, since they were able to populate three entire feathers from a papilla (P) which also contained (or had contained just previously) genetically coloured melanoblasts.

The reappearance of white in follicles P and R leads to an unforeseen ambiguity. Since the native dominant white melanoblasts of these papillae must have remained inactive during the growth of earlier feathers, it is also possible that coloured melanoblasts entered follicles O and Q soon after transplantation, but remained inactive until the fifth and third feathers respectively. The possibility that entry of new melanoblasts is restricted to the period immediately following transplantation cannot therefore be excluded, but there is no positive evidence to support it. The most obvious reason why melanoblasts might enter papillae immediately after transplantation, but at no other time, would be that native melanoblasts were absent or failed to survive transplantation, and this cannot have been the case in any of our transplants.

Our results show that the feather papilla does contain an effective reservoir of melanoblasts. On the other hand, this reservoir is not of an exclusive nature: melanoblasts are sometimes drawn in from outside the papilla. The transplants themselves yield no evidence as to the source of these melanoblasts beyond the fact that they come from outside the papilla, but the spontaneously mosaic follicle G shows that melanoblasts may sometimes be drawn from skin as far distant as 1 cm. from the follicle, or conceivably from deep sub-dermal tissue.

Since it has been shown that the mature feather follicle may admit melanoblasts, it would seem advisable to reappraise the view that the hair follicle, once fully developed, is closed to further entry of melanoblasts. There may be reasons of a purely anatomical nature (e.g. the fact that a hair follicle projects relatively much deeper into the dermis) why hair and feather follicles might differ in this respect, but the evidence for this view is less conclusive than is commonly supposed. The whitening of all subsequent hair-generations which results from mild freezing (Taylor, 1949) or X-irradiation (Chase, 1951) of the skin has been adduced in support, but neither of these agents has been shown to act *directly* on the pigment cells. Cohen (1957) has obtained evidence that the whitening of feathers following X-irradiation is mediated by a change in the nature of the 'cue' given by the epidermis to the pigment cells. Similarly, the results of skin transplants between the black and white areas of hooded rats (Rawles, 1955) and of spotted guinea-pigs (Billingham & Medawar, 1948, 1950) are inconclusive, since it is uncertain whether the black-*versus*-white difference in these animals is due to an inherent difference between the melanoblasts of the different regions, or (as in the case of the black-and-white Light Sussex fowl—Cohen (1957)) to a difference in the epidermis, causing a different cue to be given to the pigment cells. Silvers & Russell (1955) whose skin homografts in mice certainly involved two inherently (genetically) different kinds of melanoblast, reported that the number of hairs with foreign pigmentation frequently increased with the passage of time. This increase was at least in part due to the retention of more than one hair in the same follicle, but it is also open to the interpretation that there was an increase in the number of follicles containing foreign melanoblasts.

The black marginal bands occurring in our mosaic feathers on ♀ 3780/4 suggest that some substance (e.g. an enzyme, or an unused substrate) has been diffusing over from the area populated by dominant white (*I*) melanocytes into that occupied by coloured ($+^I$) melanocytes. It is perhaps significant in this respect that the homograft carries, besides *I*, the gene *E*, which in the absence of *I* would give rise to solid self-black pigmentation. (The homograft also carries *B* and *Bl*; hence the rather diffuse blue-grey pigmentation of its ticks.) The assumption that $+^E$ melanocytes are not fully autonomous in small areas would also explain why there is no clear-cut ticking effect associated with heterozygosity at the *E*-locus (Cock, unpublished observations). This is the first evidence of a phenomenon of this kind in feathers, but similar cases of imperfect autonomy, resulting in marginal effects in mosaics, have been found at certain loci in several insect species. In particular the *Habrobracon* mosaics described by Whiting (1934) provide a close analogy with our mosaic feathers.

One further point of interest arises in connexion with the mosaic feathers. Follicles G and P have each produced three mosaic feathers in succession, and in each case the three feathers have an approximately similar pattern of mosaicism. The similarity is not exact, but is too close to be entirely due to chance. The same tendency for the pattern of mosaicism to be repeated has been noted by Danforth (1929, 1939). It would thus appear that the melanoblast reservoir within the papilla has a certain degree of spatial organization, in that descendants of the melanoblasts producing the melanocytes of a particular region of one feather will tend to produce the melanocytes of the same region in the next feather. This organization is of course not permanent; it is subject to fluctuations, and to more radical alteration whenever previously inactive melanoblasts (from inside or outside the papilla) are brought into play. It is easy to imagine a mechanism which would account for the repetition of longitudinal melanocyte-mosaics (e.g. feathers with white lateral edges and black-red centre, as in follicle G), but the repetition of transverse melanocyte-mosaics (e.g. black-red feathers with a white patch at the tip, as in follicle P) presents greater difficulties. Similar difficulties arise in connexion with the repetition of structural faults, and of the structural (epidermal) mosaics produced by Lillie & Wang (1943). Cohen (1957) has proposed a theory of the mode of formation of the feather-collar from the top of the papilla, which would resolve at least some of these difficulties in the case of epidermal mosaics and structural faults. In order to account for the repetition of melanocyte-mosaics, it would be necessary to make the additional assumption that melanoblasts, and the melanocytes derived from them, tend to remain associated with a particular region of the epidermis, both in the papilla itself and during the process of collar formation.

## SUMMARY

1. In an adult black-red hen which, when a newly hatched chick, had received a homograft of skin from the back of a dominant white chick, active feather papillae have been exchanged between follicles of the neck and the homograft. The distinctive structure of feathers of the two tracts permits reliable identification of feathers growing from these transplanted papillae.

2. Each of six successful transplants from the neck into the homograft has so

far produced only black-red feathers (one to three feathers in each case). Five transplants from the homograft into the neck have so far produced from two to five feathers each, with the following sequences of pigmentation types: white—white—white—white—mosaic; black-red—black-red—mosaic—mosaic—mosaic; black-red—white—white—white—black-red; white—white—black-red; white—white. A follicle on the homograft, about 1 cm. in from the edge, and not subjected to any operative interference, has produced three mosaic feathers in succession, each partly pigmented by host-type (black-red) melanocytes.

3. It is concluded (*a*) that the papilla of a feather follicle contains a reservoir of melanoblasts sufficient to provide the melanocytes of several future feathers; (*b*) that this reservoir is from time to time augmented by melanoblasts migrating in from the surrounding tissue; and (*c*) that some of the melanoblasts within a papilla may remain inactive during the growth of one or more feathers, but contribute melanocytes to subsequent feathers.

4. In the vane of each of the seven feathers which are mosaic in respect of their melanocytes, there is a marginal band of intense black where the black-red area adjoins the white. This, it is suggested, may be due to the diffusion of some substance from the white area, and may depend on the fact that the dominant white melanocytes also carried the gene for extended black.

5. Our findings are discussed in relation to previous work on the pigmentation of feathers and hairs.

## ACKNOWLEDGEMENTS

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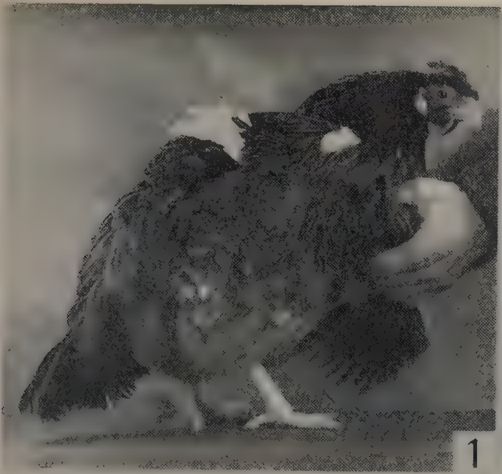
## EXPLANATION OF PLATES

### Plate 1

Fig. 1. Photograph of ♀ 3780/4, taken on 21 February 1957. The homograft is visible as a large patch of white plumage at the anterior end of the saddle. Immediately behind it is a control autograft, taken from the site now occupied by the homograft. Both grafts are oriented transversely, with their morphologically anterior ends towards the right of the host. The single white feather visible near the base of the neck is Q 2 (see Table 1, and Plate 2, fig. 7).

Figs. 2, 3, and 4. Typical feathers from unoperated follicles on ♀ 3780/4: 2, neck; 3, saddle; 4, saddle homograft. Note the characteristic differences in structure and shape between neck and saddle feathers. In saddle feathers the fluff extends nearly two-thirds of the length of the feather;



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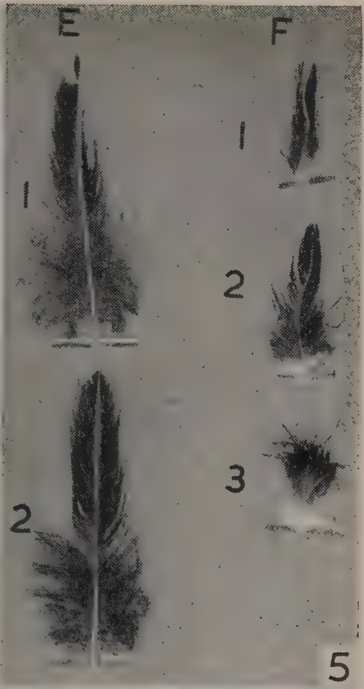

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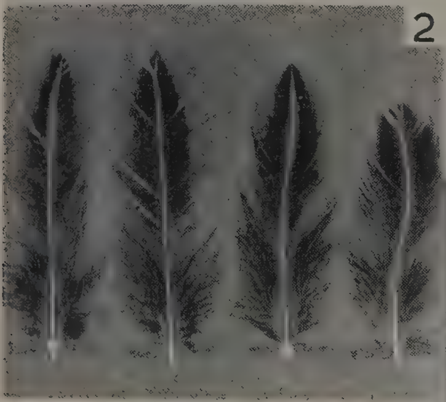

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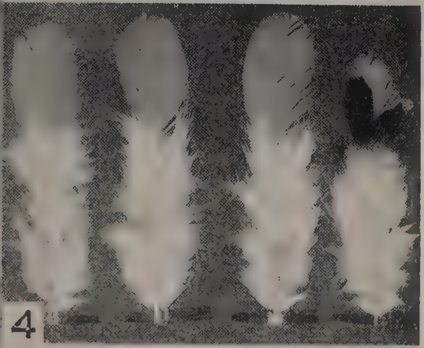

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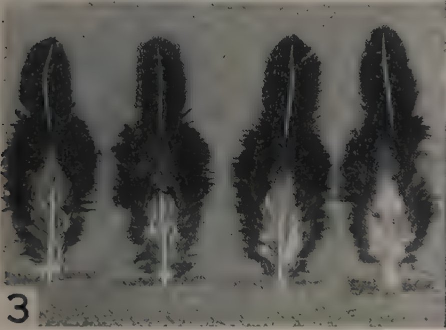

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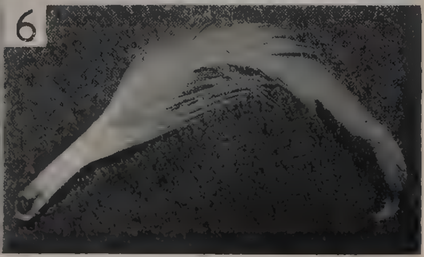
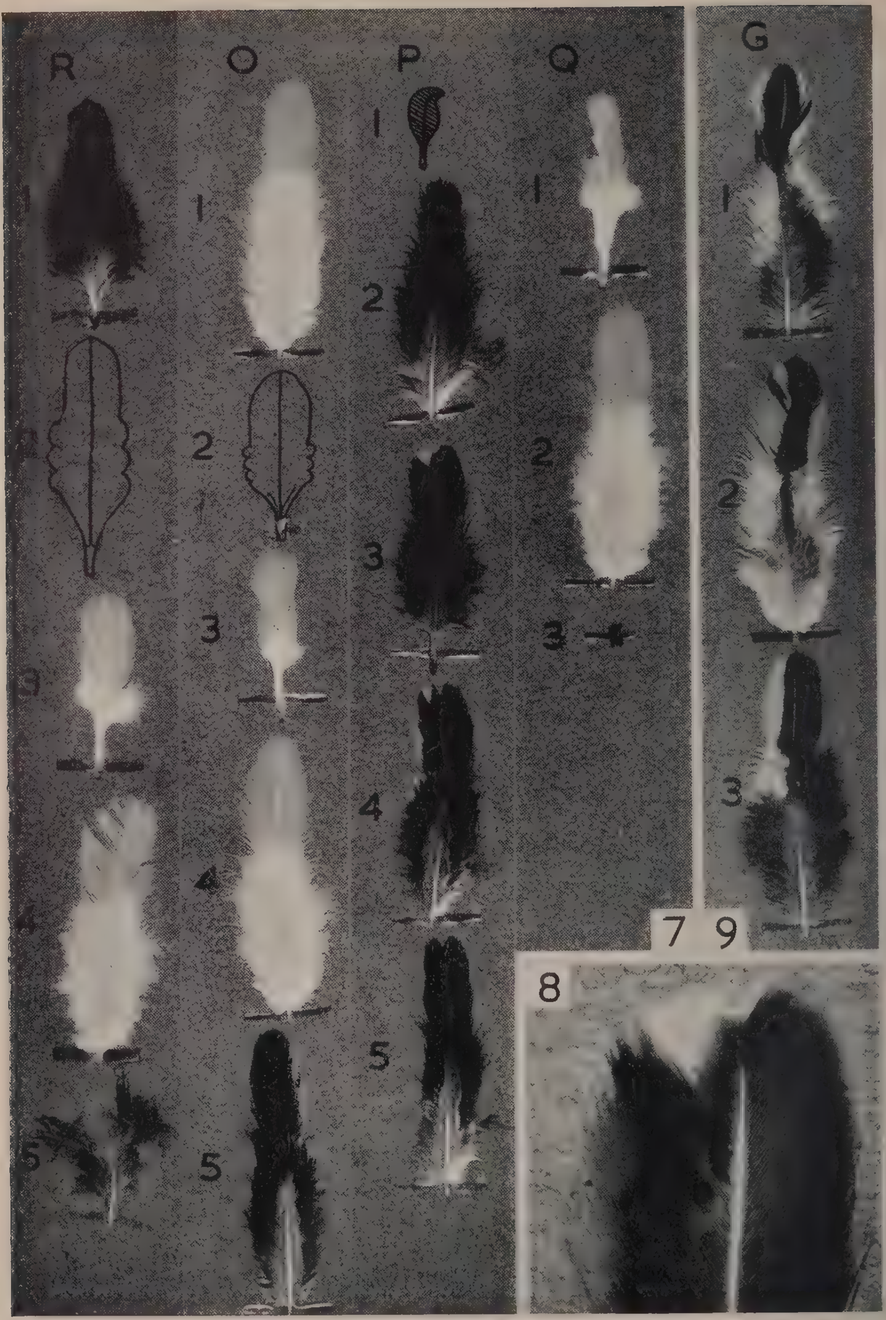


6

A. G. COCK *and* J. COHEN

*Plate 1*



A. G. COCK *and* J. COHEN

*Plate 2*

in neck feathers, it is confined to the basal third. The vane of a neck feather has a much wider barbuleless fringe, and a slightly less rounded tip. Note also the quite distinct pigmentation patterns in 2 and 3; the neck feathers have more brown and less black. One of the homograft feathers (4) has a 'tick' (see text) of rather diffuse blue-grey; *no* brown pigment is present in this. ×0·5.

FIG. 5. Some of the feathers grown from neck papillae of ♀ 3780/4 transplanted into the homograft (see Table 1). E 1 and E 2 are clearly neck type in both structure and pigmentation pattern (cf. fig. 2). F 1, F 2, and F 3 are all severely dwarfed, in spite of which F 2 shows a close approximation to neck-type structure and pigmentation. ×0·5.

FIG. 6. Feather grown from a breast papilla of ♂ 3774/4 transplanted into the homograft. Most of the feather is white and has the typical structure of a ♂ saddle feather, but there is a small area of black-and-brown pigmentation at the extreme tip. The tip of the feather is curled and rather defective, but appears to be breast-type in structure in that barbules are present along almost the whole length of the barbs. The area of apparent breast structure is a little more extensive than the pigmented area. Presumably both the melanoblasts and the epidermis of the transplanted papilla were replaced by cells from the surrounding homograft tissue soon after the start of growth of this feather. ×1·4.

## PLATE 2

FIG. 7. Some of the feathers grown from saddle homograft papillae transplanted into the neck (see Table 1). Feathers which were lost before plucking are drawn in diagrammatically, white feathers being drawn in outline only, and coloured feathers being cross-hatched. Note that all feathers, whether white or coloured, have the characteristic saddle-type shape and structure, and that the coloured ones have saddle-type pigmentation pattern. O 5, P 3, P 4, and P 5 are all mosaic, with small patches of white in the vane. ×0·5.

FIG. 8. The tip of feather P 3 magnified to show the black marginal band where the white and coloured parts adjoin. ×1·7.

FIG. 9. Three spontaneously mosaic feathers grown from a follicle (G) on the saddle homograft of ♀ 3780/4 (see Table 1). Note the pale brown rachis-stripe and the predominantly black pigmented parts of the vane: these features clearly differentiate the feathers from the occasional ticked feathers on the homograft (cf. Plate 1, fig. 4). ×0·5.

*(Manuscript received 24 : iii : 58)*

*Note added in proof—23 October 1958*

The following feathers were today plucked from ♀ 3780/4; none of them was fully grown.

On the homograft: A 2: neck-type structure, black-red pigmentation. D 2: defective structure, black-red. E 3: *saddle*-type structure, white, orientation approximately normal. (From the position relative to tattoo-marks it is almost certain that this feather grew from follicle E, in which case it must derive from a papilla regenerated from homograft tissue.) F 4: two feathers growing in the same follicle; one neck-type, the other defective in structure, both black-red. H 1: defective structure, black-red. (H presumably contains a papilla transplanted from the neck, but no feathers have previously been observed to grow from it.)

On the neck: O 6: saddle-type structure, mosaic pigmentation (mainly black-red, with white tip and pronounced black marginal band). Q 4: defective structure, black-red. R 6: two feathers growing in the same follicle (cf. the partially twinned R 5); one is saddle-type and white, the other dwarfed and defective in structure and black-red.

These new feathers reinforce the general conclusions previously drawn. Apart from the very dubious case of E 3, there is still no evidence of dominant white melanoblasts entering neck papillae transplanted into the homograft.

# The Mechanism of Hardening of the Salmonid Egg Membrane after Fertilization or Spontaneous Activation

*by* A. I. ZOTIN[1]

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THE embryonic development of fishes proceeds under the protection of rigid egg membranes which preserve the embryo from mechanical injury. Salmonid fishes bury their eggs in sandy and stony ground so that they are particularly liable to mechanical damage. This is apparently the reason why the membrane of the salmonid embryo is extremely strong, resisting a load of 3–4 kg. per ovum (Gray, 1932; Hayes, 1942, 1949; Hayes & Armstrong, 1942; Zotin, 1953*a*). This mechanical property of the membrane does not appear immediately after fertilization or activation but is preceded by a whole set of processes which are elicited in the membrane by external factors and by the fertilized or activated egg itself. Thus, according to Manery, Fisher, & Moore (1947), hardening of the egg membranes in the speckled trout sets in 2 hours after the release of the egg into water. Ca ions have been shown to be of great significance for membrane-hardening in *Salvelinus fontinalis* (Manery, Fisher, & Moore, 1947; Warren, Fisher, & Manery, 1947; cf. also Hoar, 1957) and *Oncorhynchus keta* (Kusa, 1949 *a*, *b*). In distilled water, membrane-hardening is slower than in ordinary water and is completely blocked when Ca ions are bound by 0·01 N sodium citrate or 0·02 N sodium oxalate.

Fertilized eggs of the lake salmon and trout have been shown by Zotin (1953*a*, 1958) to secrete substances which are indispensable for the increase of membrane toughness. The secretion of these substances requires a short exposure to water to activate the egg. The activating effect of water upon unfertilized salmon eggs has often been described (K. Yamamoto, 1951; Soin, 1953; Disler, 1954).

The secretion of substances eliciting hardening or solidification of egg membranes after fertilization or activation has been described in some sea-urchin species (Motomura, 1941; Runnström, 1947, 1952) and in sturgeons (Zotin, 1953*b*) as well as in the teleostean fish *Oryzias latipes* (Nakano, 1956). Solidifi-

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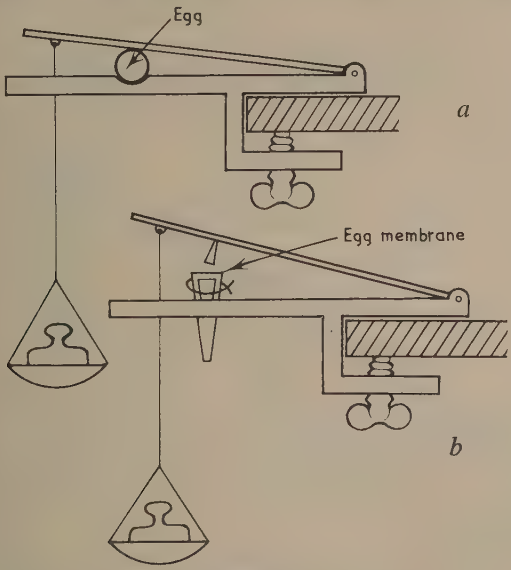
cation of the membranes of the sea-urchin egg likewise requires the presence of Ca ions (Hobson, 1932).

The following conclusions may be drawn from the above evidence with regard to membrane-hardening: (1) it is preceded by the release from the egg of substances; (2) it requires the presence of Ca ions in the medium; (3) it does not begin immediately after fertilization. A detailed consideration will therefore be presented of the above three items, viz., release of membrane-affecting substances from the egg; the effect of diverse external factors upon the hardening process; and, finally, the latent period of membrane-hardening.

## MATERIALS AND METHODS

The toughness of the vitelline membrane (chorion) of the following species was studied: of the lake salmon (*Salmo salar* morpha *sebago* Girard), of the lake trout (*S. trutta* morpha *lacustris* Linn.), and of the Svir whitefish (*Coregonus lavaretus baeri* natio *swirensis* Pravdin). The study was carried out in the autumn of 1951, 1952, 1956, and 1957 at the Svir fisheries. The ova were procured from females kept in tanks and were fertilized by the dry method, then incubated at 2–4°. The temperature of the laboratory varied within the range of 3–5°.



TEXT-FIG. 1. Devices for assaying membrane toughness in salmonid eggs.

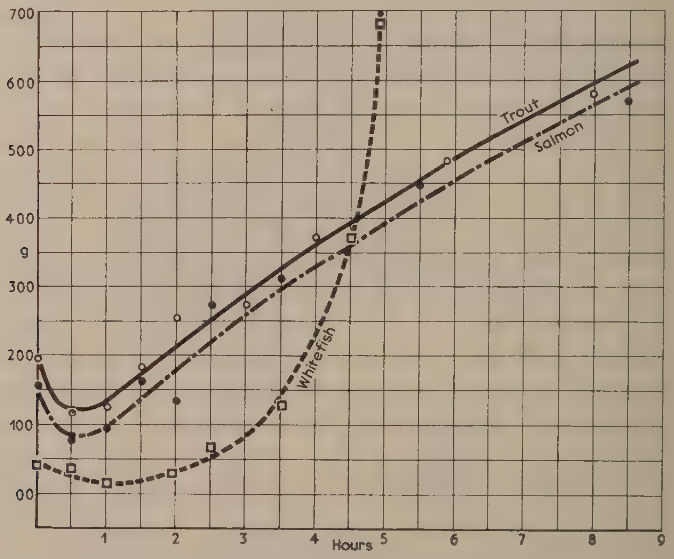
The toughness of the membrane of the salmon and trout embryos was assayed by means of the device (Text-fig. 1*a*) described by Gray (1932), estimates being made of the load necessary to squash the ovum. Another device (Text-fig. 1*b*) was used to assay rupture resistance of membranes removed from the ova (Zotin, 1953*a*). The toughness measurements of the whitefish egg membranes during the initial period when the egg can resist no more than 250 g. have been carried out by means of a device previously used for assaying the toughness of egg membranes in sturgeons (Zotin, 1953*b*). The hardness of the membranes of advanced whitefish embryos was estimated by means of Gray's apparatus. It will be noted that the above devices are based on the lever principle so that the force acting upon the membranes is to be calculated with due account of the constants of the devices.

In measuring rupture resistance of the membranes (Text-fig. 1*b*) it is possible to estimate the pressure per unit area of membrane at the moment of rupture. The area of the part of this device exerting the pressure is 0·0154 cm.$^2$ Membrane toughness assayed by means of this device was expressed in g./cm.$^2$, and in g. when Gray's apparatus was used.

## EXPERIMENTAL RESULTS

### 1. *Hardening of the vitelline membrane following fertilization or activation*

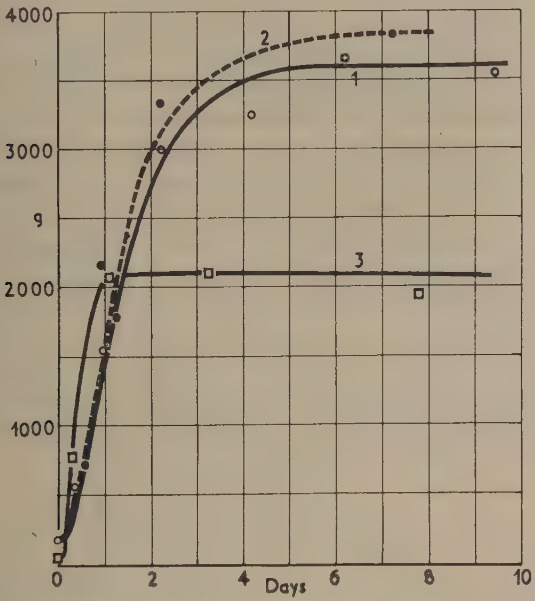
This was followed in fertilized ova of 9 female trout, 10 female salmon, and 3 female whitefish. In all cases similar data have been obtained. Unfertilized trout and salmon eggs procured from the coelomic fluid can resist as much as 160–280 g. (220 g. on the average) and those of the whitefish 40–50 g. After



TEXT-FIG. 2. Changes of egg membrane toughness in salmon, trout, and whitefish within the first few hours after fertilization (temperature 4°).

insemination the membrane toughness of eggs transferred to water at first undergoes a decrease. During this period the eggs of the trout and of salmon resist no more than 60–120 g. (average 130 g.), and those of the whitefish 20 g. After 60–120 minutes in the trout and salmon egg, and after 120–80 minutes in the whitefish egg, membrane toughness rapidly increases, reaching within 3–7 days the maximum value in the salmon and trout egg (2,500–3,500 g. load) and within 1–2 days in the whitefish egg (1,800–2,200 g.). Text-figs. 2 and 3 illustrate typical toughness curves for egg membranes of the trout, salmon, and whitefish following fertilization.

Rupture resistance of egg membranes of the trout and salmon amounts in unfertilized eggs to 1,500–2,000 g./cm.$^2$ while the maximum value in fertilized eggs is 19,000–21,000 g./cm.$^2$



TEXT-FIG. 3. Changes in membrane toughness of the unfertilized trout egg (2) upon transfer to water, and of fertilized trout (1) and whitefish eggs (3) during the first few days of development (temperature 4°).

In water-activated salmonid eggs, hardening of the membranes proceeds as rapidly as in fertilized eggs (Text-fig. 3).

An inspection of the curves presented in Text-figs. 2 and 3 shows that the increase in membrane toughness passes through the following three stages: (1) a preparatory period during the first 1–2 hours following fertilization, when membrane toughness does not increase but even decreases; (2) a period of rapid increase in membrane toughness which begins 1–2 hours after fertilization and is completed 3–7 days thereafter in salmon and trout ova, and within 1–2 days in those of whitefish; and (3) a period of maximum membrane toughness which does not undergo any further change. This is, however, only the outward picture of membrane-hardening. It is actually preceded by events which involve activation of the egg and the release of membrane-affecting substances. In addition, complex processes take place within the membranes themselves.

### 2. *The membrane-hardening enzyme*

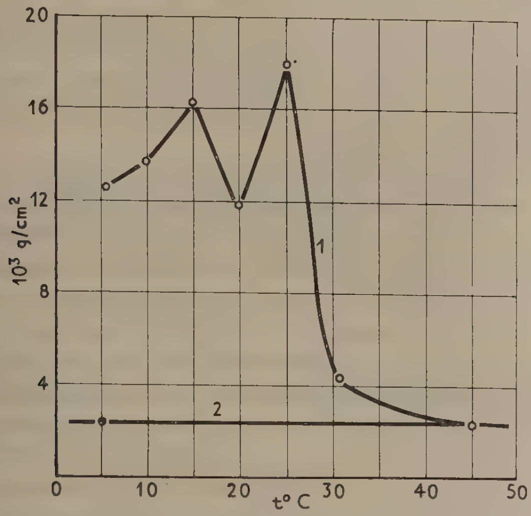
As mentioned above, the beginning of the process which causes membrane-hardening in salmonid eggs is linked with the release from the egg of membrane-affecting substances. This is suggested by toughness changes of membranes removed from non-activated eggs: hardening in these membranes proceeds at a much lower rate than in those of normally developing eggs (Zotin, 1953*a*). Direct evidence shows that the perivitelline fluid of freshly fertilized or activated salmonid eggs contains substances which call forth hardening of the membranes of non-activated eggs. The experimental procedure is as follows. Three drops of water and the perivitelline fluid of 10 trout eggs 30–60 minutes after fertilization are placed in depressions in a paraffin block (the perivitelline fluid is procured by puncturing the membrane without injuring the yolk). The membranes removed from non-activated eggs in a 0·1 M NaCl solution are placed in the depressions. The toughness of these membranes is assayed after 10 hours by means of the device presented in Text-fig. 1*b*. As controls, membranes removed from non-activated eggs are used, as well as those removed 10 hours after fertilization when tests were made of all the experimental membranes. Further, the activity of the perivitelline fluid was tested 27 hours after its removal from the fertilized eggs, and that of the perivitelline fluid of control eggs 27 hours after fertilization.

TABLE 1

*Effect of perivitelline fluid of the trout egg upon the membranes removed from unfertilized eggs*

| *No.* | *Condition of egg from which the membrane has been removed* | *Medium in which removed membranes are placed* | *Toughness of membranes after 10 hours (in g./cm².)* | *Average of* |
|---|---|---|---|---|
| 1 | Non-activated | water | 3,800 | 10 |
| 2 | Ten hours after fertilization | water | 9,000 | 10 |
| 3 | Non-activated | perivitelline fluid from eggs 30–60 min. after fertilization | 14,000 | 10 |
| 4 | Non-activated | same perivitelline fluid 27 hours after procuring | 5,000 | 3 |
| 5 | Non-activated | perivitelline fluid from eggs 27 hours after fertilization | 5,800 | 3 |

It will appear from the data of Table 1 that the perivitelline fluid of the trout contains a substance which causes membrane-hardening (Table 1, row 3) and that it is partially inactivated after 27 hours both in water (row 4), and in the perivitelline fluid of intact eggs (row 5). Repeated experiments showed that this substance is not inactivated after 4 and 8 hours (at 5·7°) but is inactivated after 30 hours. The substance does not pass across the egg membrane and is inactivated by a 5-minute exposure to 30° of the non-diluted perivitelline fluid from fertilized eggs 30–60 minutes after fertilization (Text-fig. 4).

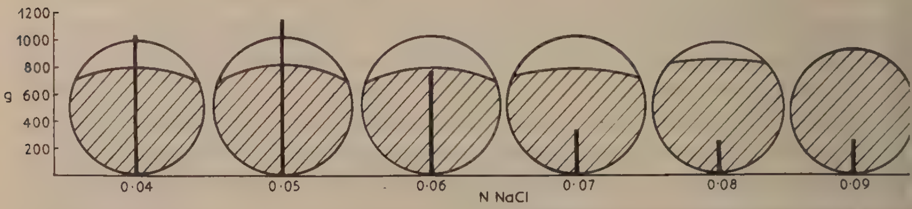


TEXT-FIG. 4. The effect of temperature (5-minute heating) upon the capacity of the perivitelline fluid of salmon eggs to cause hardening of membranes removed from non-activated eggs. (1) Toughness of membranes treated with perivitelline fluid; (2) toughness of membranes not so treated. (Each point is the average of three measurements.)

The membranes of the salmonid eggs are known to be readily permeable to water, salts, and highly dispersed colloids, but impermeable to low-dispersed colloids (Svetlov, 1928, 1929; Bogucki, 1930; Gray, 1932). Hence the substance released by the salmonid ovum after fertilization and causing hardening of the membranes is an unstable high molecular compound rapidly inactivated by high temperature, presumably an enzyme. It will be noted that the hardening enzyme can apparently be inactivated while still in the cytoplasm of the egg. Thus, the membranes of unfertilized salmon eggs kept in liquid paraffin for 27 days did not harden upon transfer to water although the perivitelline space and the blastodisc formed as normally.

The release of substances from the cortical layer of fish eggs after fertilization has been described by numerous authors and shown to participate in the formation of the perivitelline space (for literature see T. Yamamoto, 1939 *a*, *b*, 1954, 1956; Kryžanowski, 1953, 1956; Kusa, 1953*b*, 1956; Thomopoulos, 1953 *a*, *b*; Dettlaff & Ginsburg, 1954; Zotin, 1953*a*, 1954, 1958; Dorfman, 1955; Dettlaff, 1957*a*). These substances are represented in the cortical cytoplasm of unfertilized eggs by special structures (these have been given different names, such as vesicles, droplets, platelets, granules, or alveoli) which disappear from the cortical layer after fertilization or activation concurrently with the formation of the perivitelline space. In salmonid eggs such structures have been described by Kanoh (1950), K. Yamamoto (1951), Kusa (1953*a*, 1954, 1956), Devillers, Thomopoulos, & Colas (1954). The cortical alveoli of fish eggs were shown to contain, in addition to a small amount of protein, mostly mucoproteins with acid polysaccharides (Kusa, 1953 *a*, *b*, 1954, 1956; Thomopoulos, 1953*a*; Aketa, 1954; Nakano, 1956; Dettlaff, 1957*a*).

The question arises as to whether the hardening enzyme is contained in the cortical alveoli and is released concurrently with the secretion of their contents beneath the membrane. It has been suggested by Nakano (1956) that hardening of egg membranes of *Oryzias* is elicited by some component substances of the cortical alveoli. This does not, however, seem to be the case in salmonid eggs (Zotin, 1958). The disappearance of cortical alveoli and the formation of the perivitelline space are known to be blocked in the salmonid eggs by NaCl and by some other salts (Bogucki, 1930; Devillers, Thomopoulos, & Colas, 1954; Kusa, 1953*a*, 1956; Zotin, 1954). Hence, by blocking the release of substances which call forth the formation of the perivitelline space or by inducing their release it should be possible to identify the hardening enzyme with or distinguish it from these substances by simultaneously assessing the toughness changes in the membranes. Several experimental series have therefore been carried out on the effect of various NaCl concentrations on the formation of the perivitelline space and membrane-hardening in fertilized salmon, trout, and whitefish ova. The latter were transferred from the coelomic fluid to the saline with no water as intermediate (in these experiments as in all others the salt solutions were prepared in river water). It proved possible to adjust NaCl concentration so that although the perivitelline space is formed the membrane toughness did not differ from that of the unfertilized egg (Text-fig. 5).



TEXT-FIG. 5. The effect of NaCl concentration upon formation of the perivitelline fluid and hardening of salmon egg membranes.

Formation of the perivitelline space without subsequent hardening of the membranes is noted in eggs activated by water for 2–3 minutes and then transferred to a 0·1 N NaCl solution. The perivitelline space is formed in unfertilized eggs transferred to 0·1 N LiCl although in this solution no hardening of the membranes takes place. Finally, the perivitelline space is formed in unfertilized eggs kept in liquid paraffin for 27 days and then transferred to water, although no hardening of the membranes takes place.

TABLE 2

*Effect of NaCl upon the activity of the hardening enzyme of the perivitelline fluid of salmon egg*

| *Membranes removed from non-activated eggs and placed in* | *Toughness of such membranes in g./cm.² (average of 3)* | |
|---|---|---|
| | *After 19 hours (expt. No. 1)* | *After 27 hours (expt. No. 2)* |
| Mixture (1 : 1) of perivitelline fluid and 0·2 N NaCl solution | 7,500 | 13,800 |
| Mixture (1 : 1) of perivitelline fluid and water | 9,400 | 12,300 |
| Water | 1,900 | 3,600 |

It can be shown that NaCl blocks the secretion of the enzyme from the egg but does not inhibit its effect upon the membranes. It will appear from the data of Table 2 that the perivitelline fluid containing the enzyme when added to a 0·2 N NaCl solution (final NaCl concentration 0·1 N) affects isolated membranes in the same way as water-diluted perivitelline fluid. In the experiments illustrated in Text-fig. 5 the hardening enzyme must therefore be lacking in the perivitelline fluid, since the saline cannot prevent its effect upon the membranes. Thus the hardening enzyme of the salmonid egg has no connexion with the substances which take part in the formation of the perivitelline fluid. Since it is claimed by most workers that the formation of the perivitelline fluid is associated with disintegration and disappearance from the egg surface of cortical alveoli it may be concluded that the hardening enzyme is not contained in the cortical alveoli of the non-activated egg.

### 3. *Duration of action of the hardening enzyme upon the membrane*

Fertilization or spontaneous activation of the salmonid egg is accompanied by the formation of the perivitelline space, hardening of the membrane, and formation of the blastodisc and of the oil zone. Although all these changes appear soon after fertilization or activation they are to some extent independent of one another. Thus Kusa (1953 *a*, *b*), Thomopoulos (1953 *a*, *b*), Devillers, Thomopoulos, & Colas (1954) report normal formation in certain salt solutions of the blastodisc and of the oil zone (bipolar differentiation) but not of the perivitelline space. As already pointed out, membrane hardening and formation

TABLE 3

*Exposure time of salmon eggs to water necessary for the release of the hardening enzyme*

| | *Unfertilized eggs* | *Fertilized eggs* | | | | | | | | | | | *Control (fertilized eggs at the moment toughness was measured)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exposure time in water (in min.) prior to transfer to 0·1 N NaCl solution or oil . . . | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 3 | 5 | 8 | 10 | 12 | 15 | 20 | |
| Toughness (in g.) of membranes of eggs exposed to 0·1 N NaCl — 8 hours | 200 | — | — | — | 120 | — | 1,000 | — | 1,200 | — | 1,400 | — | 990 |
| Toughness (in g.) of membranes of eggs exposed to 0·1 N NaCl — 4 hours | 90 | 100 | 80 | 120 | — | 360 | 340 | — | — | — | — | — | 360 |
| Toughness (in g.) of membranes of eggs exposed for 14 hours to liquid paraffin . . . | 280 | — | — | — | — | 180 | — | 1,000 | — | 3,400 | — | 2,000 | 960 |

of the perivitelline space can likewise be dissociated from one another. It has been mentioned above that a short exposure of the unfertilized egg to water is necessary for the release of the hardening enzyme as well as for the formation of the perivitelline space and bipolar differentiation. The exposure time is, however, different for the secretion of the enzyme and for that of the substances eliciting the formation of the perivitelline space. Thus, if salmon eggs are placed for a short time in water and then transferred to 0·1 N NaCl (which in the unfertilized egg blocks the release of the enzyme and of the substances from the cortical alveoli) 30–60 seconds in water will suffice to induce the formation of the perivitelline fluid, whereas hardening of the membrane requires a 2–3 minute exposure (Table 3).

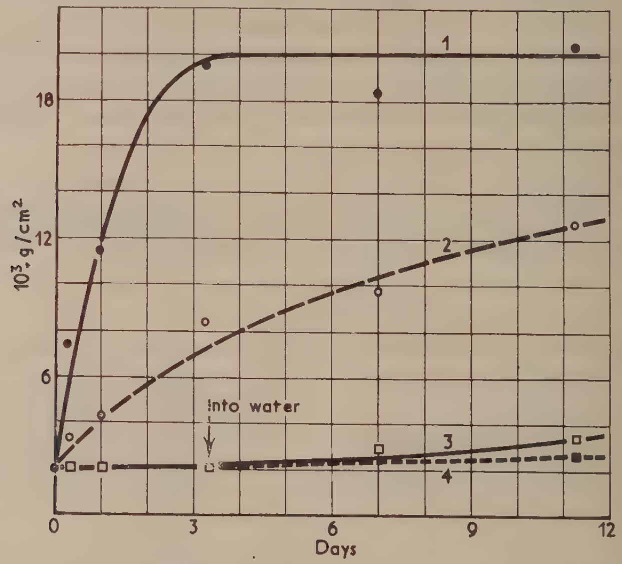
It is possible not only to evaluate the time of action of water which is necessary for the secretion of the hardening enzyme but also to determine the total time the egg should be present in order that hardening of the membrane be initiated. For this purpose the membranes are removed from the eggs at certain time intervals following fertilization and their toughness is assayed. Table 4 shows that if the membranes are removed from the eggs 6–7 minutes after transfer to water their toughness either remains unchanged or changes at a slower rate than that of the membranes of intact eggs. Since activation of the eggs and the release of the enzyme require 2–3 minutes (Table 3), the duration of enzyme action upon the membranes is 3–4 minutes at 4°. Then the increase in membrane toughness continues in the absence of the enzyme.

TABLE 4

*Exposure time necessary for enzyme action upon membranes*

| *Time of removal of membranes after fertilization (in min.)* | *Membrane toughness in g./cm.²* | |
|---|---|---|
| | *trout (after 9 hours)* | *salmon (after 10 hours)* |
| Unfertilized eggs | 3,700 | 8,700 |
| 6 | — | 10,500 |
| 7 | 3,300 | — |
| 12 | — | 15,700 |
| 15 | 13,000 | — |
| 20 | — | 19,000 |
| 25 | 14,300 | — |
| 35 | — | 19,300 |
| Control (membranes removed at the moment of toughness test) | 10,100 | 14,800 |

The question arises whether the effect of the enzyme upon egg membranes is indispensable for the hardening process or whether it merely accelerates a slowly progressing process in the membrane itself. The latter alternative would appear more plausible since in membranes removed from unfertilized eggs and transferred to water hardening, although slow, goes on (cf. Table 4 and Text-fig. 6, curve 2). It must, however, be remembered that while the membranes are being removed from unfertilized eggs in the coelomic fluid (and this is carried out by squeezing the egg contents through a rupture in the membrane) part of the egg contents remains in the membrane. It is extremely difficult to remove the egg remnants from such a membrane in water because of coagulation of the yolk which adheres to it. Along with the egg remnants the hardening enzyme probably also adheres to the membrane. If, however, membranes removed in the coelomic fluid are transferred to a solution of 0·1 N NaCl instead of water, the yolk remnants can easily and fairly rapidly be eliminated. In such membranes no increase in toughness occurs either when kept in 0·1 N NaCl (Text-fig. 6, curve 3) or upon transfer from the saline to water (Text-fig. 6, curve 4). Thus, it may be concluded that in the salmonid embryo the enzyme is indispensable for membrane-hardening.



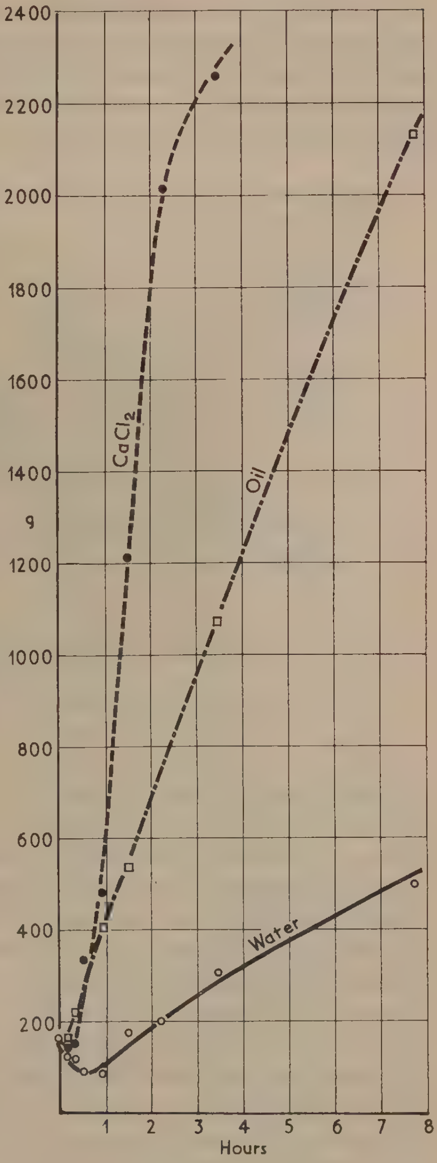
TEXT-FIG. 6. Change in membrane toughness of normally developing salmon eggs (1), of membranes removed from non-inseminated eggs and placed in water (2), and in 0·1 N NaCl solution (3). Some of the membranes transferred from 0·1 N NaCl solution to water (4). (Each point is the average of five measurements.)

### 4. *Factors regulating the rate of membrane-hardening*

*Water.* As mentioned above, water activates salmonid and whitefish ova and in this respect it is an indispensable link in the processes preceding membrane-hardening, since no hardening enzyme is secreted unless the eggs are treated for 2–3 minutes with water. It has been suggested by Manery & Irving (1935), and some indirect evidence pertaining to sturgeons corroborates this suggestion (Zotin, 1953*b*), that the water participates in the hardening process as well. If this be so, it is a matter of the very first few minutes, since already 2–3 minutes after exposure to water transfer to a non-aqueous medium (liquid paraffin) does not prevent the course of the hardening process (Table 3). It thus appears that water in the surrounding medium calls forth the release of the hardening enzyme from the salmonid and whitefish egg and possibly participates in the very first processes preceding membrane-hardening.

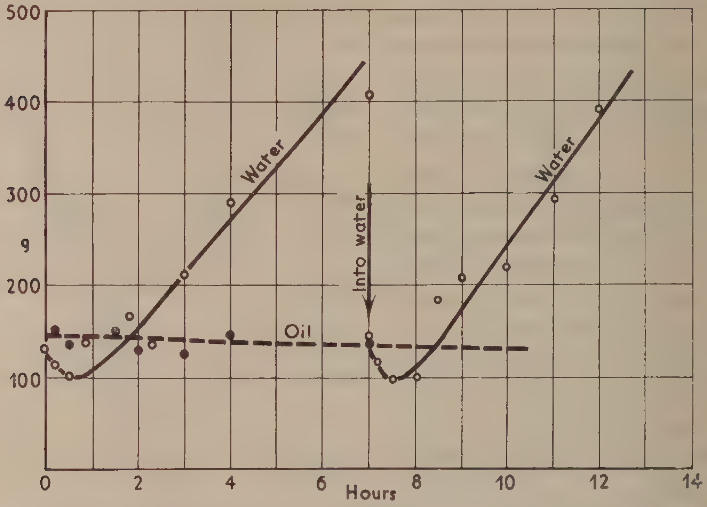
At more advanced stages water in the medium plays an opposite role in the hardening processes, as a factor regulating (inhibiting) their rate. If the salmon egg be placed into a non-aqueous medium, such as liquid paraffin, after a 5–10-minute exposure to water (to bring about activation), membrane-hardening will proceed at a higher rate than in control ova exposed to water (Text-fig. 7).

In these experiments liquid paraffin presumably plays the role of a non-aqueous medium, but not of a chemical stimulus of the hardening process. Indeed, if unfertilized salmon eggs are placed in liquid paraffin without water as intermediate the toughness of the membranes does not increase during a considerable lapse of time (in one experiment up to one month). When, however, these eggs are exposed to water, normal membrane-hardening occurs (Text-fig. 8). Moreover, the stimulating effect of liquid paraffin on the hardening pro-



TEXT-FIG. 7. The effect of liquid paraffin and of 0·1 N $CaCl_2$ solution on the rate of toughening in salmon egg membranes following fertilization. Prior to transfer to paraffin and $CaCl_2$ solution the eggs were placed for 5 minutes in water (for activation). (Each point is the average of five measurements.)

cess is noted only if the eggs are transferred to the paraffin within the first 20–25 minutes after fertilization or activation (Table 5). Hence, the effect of liquid paraffin on membrane-hardening is due to the fact the egg is placed in a non-aqueous medium. It is noteworthy that water passage into the perivitelline space is most intense during the first 20–25 minutes after fertilization while this space is being formed (Bogucki, 1930; Hayes & Armstrong, 1942; Privolnev, 1952; Zotin, 1954). It may therefore be assumed that it is passage of water across the membrane during the formation of the perivitelline space that regulates the rate of membrane-hardening by slowing up this process.



TEXT-FIG. 8. Change in membrane toughness of unfertilized salmon eggs in water; liquid paraffin; and water after a 7-hour exposure to liquid paraffin.

*Salts*. It has been shown above (Text-fig. 5) that 0·1 N NaCl solution blocks the release of the hardening enzyme from the unfertilized ovum. A similar effect upon unfertilized salmonid eggs is produced by many other salts (Table 6). The effect of 0·1 N solutions of NaCl, KCl, $CaCl_2$, and $MgCl_2$ upon unfertilized eggs consists in blocking the secretion of the hardening enzyme: the solutions of these salts do not inhibit the hardening process itself when the eggs are placed at first for 10 minutes in water, to activate and release the enzyme, and then transferred to the salt solution (Table 6). This is not, however, the case with 0·1 N solutions of LiCl, $AlCl_3$, and $FeCl_3$ which inhibit the hardening process even after the egg is activated and the enzyme released beneath the membrane. LiCl, $AlCl_3$, and $FeCl_3$ have not been tested in buffered solutions, so it is difficult to decide whether hardening is affected by the ions of these salts or by the reaction of the medium.

It appears from the data of Table 6 and Text-figs. 7, 9, and 10 that divalent

TABLE 5

*Time during which oil acts as an accelerator of membrane-hardening (salmon eggs)*

| *Exposure time of eggs to water after fertilization but prior to transfer to oil (in min.)* | | *5* | *8* | *10* | *12* | *15* | *20* | *25* | *30* | *40* | *60* | *Control (egg in water)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Membrane toughness (in g.) | After 9 hours (liquid paraffin) | 1,800 | — | 1,600 | — | — | 1,000 | — | 700 | — | — | 670 |
| | After 5 hours (linseed oil) | 2,000 | 1,400 | 1,300 | 1,300 | 650 | 900 | 550 | 300 | 150 | 150 | 250 |

TABLE 6

*Effect of salts on membrane toughness of* (1) *activated (10 min. exposure in water) and* (2) *non-activated trout eggs*

| | | *0·1 N solution* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *LiCl* | *NaCl* | *KCl* | *$CaCl_2$* | *$MgCl_2$* | *$SrCl_2$ ($10^{-3}m$)* | *$AlCl_3$* | *$FeCl_3$* | *$H_2O$* |
| Membrane toughness (in g.) 6 hours after transfer of eggs to the solution | (1) | 90 | 610 | 520 | 1,920 | 990 | 2,270 | 61 | 100 | 400 |
| | (2) | 90 | 130 | 110 | 95 | 82 | — | 47 | 47 | 400 |

Ca, Mg, and Sr ions greatly accelerate the increase in membrane toughness, provided they act after activation of the egg and release of the hardening enzyme. A similar although much less pronounced effect is produced by monovalent K and Na ions. Acceleration or inhibition of the hardening process within the first hours following fertilization or activation does not necessitate continuous action of the salts upon the membranes. In fact, a 10-minute (or even less) exposure of the eggs to 0·1 N salt solutions will suffice to reveal their effect upon the hardening process.

TABLE 7

*The significance of Ca ions for the effect of the hardening enzyme upon membranes of salmon eggs removed from non-activated eggs*

| *Membranes removed from non-activated eggs in 0·1 N solution of* | *Transferred* | *Membrane toughness after 20 hours, in g./cm.² (average of three)* |
|---|---|---|
| NaCl . . . . | for 10 min. to 0·1 N versene solution and thereafter for 5 min. to water and enzyme solution | 3,500 |
| Versene . . . . | for 5 min. to water and enzyme solution | 2,100 |
| Versene . . . . | for 30 min. in 0·1 N $CaCl_2$ solution rinsed in water and to enzyme solution | 6,300 |
| NaCl . . . . | to enzyme solution | 11,400 |
| Controls . . NaCl | to 0·1 N NaCl solution | 1,600 |
| Versene | to 0·1 N versene solution | 2,200 |

*Calcium.* As mentioned above, like other salts of bivalent metals, 0·1 N $CaCl_2$ prevents the release of the enzyme from the surface of the unfertilized egg, thereby blocking membrane-hardening, but it greatly accelerates the hardening process in fertilized or activated eggs (Text-fig. 7). Moreover, Ca ions play another, more specific, role in membrane-hardening. According to Manery, Fisher, & Moore (1947), Warren, Fisher, & Manery (1947), and Kusa (1949*b*), membrane-hardening of the salmonid eggs does not proceed in a Ca-free medium. When eggs of the Svir salmon and trout are placed in solutions of 0·1 N oxalate or versene which completely bind Ca ions, no membrane-hardening will occur (Dettlaff and Zotin, 1958). Table 7 contains data illustrating the effect of 0·1 N versene upon isolated membranes removed from unfertilized eggs. The hardening enzyme does not cause hardening of the Ca-free membranes. It will be noted that Ca ions are necessary for membrane-hardening only during the first 5 minutes after fertilization or activation, that is to say, while the enzyme acts upon the membrane; after this, hardening is even accelerated in a Ca-free medium (cf. Text-fig. 10). Ca ions are therefore necessary for enzyme action but not for the hardening process itself.

There are abundant data in the literature on the role of Ca ions in activation of the egg (Heilbrunn, 1937; Runnström, 1949, 1952; T. Yamamoto, 1954; Yanagimachi & Kanoh, 1953; Dorfman, 1955; Dettlaff, 1957*b*). Kusa (1950) and Kanoh (1952) did not succeed in demonstrating the participation of Ca ions in activation of salmonid eggs. However, by means of versene, which has a greater Ca binding capacity than citrate or oxalate, it has been shown (Dettlaff, 1958) that Ca ions play the same role in the activation of salmonid eggs as in other fish species. Since activation of the egg is effected with the aid of Ca ions it may be inferred that the release of the enzyme may also necessitate the assistance of these ions.

TABLE 8

*Significance of oxygen for membrane-hardening in the trout egg*

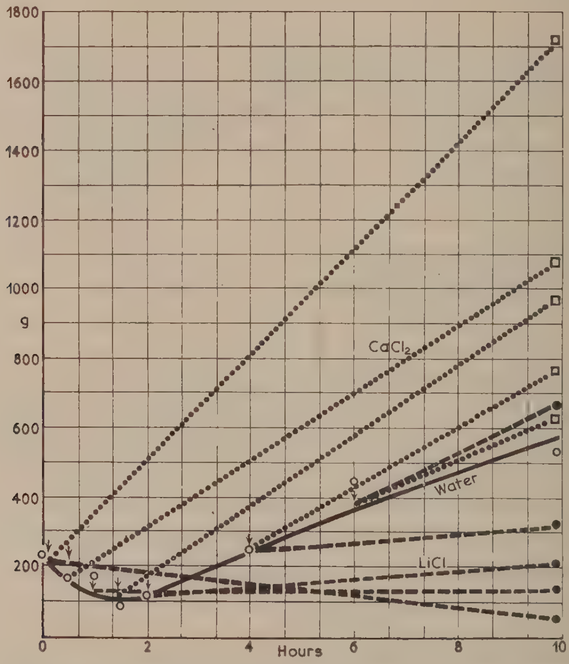
| *Eggs placed in* | | *Membrane toughness (in g.) of these eggs after 4 hours (average of five)* |
|---|---|---|
| Ordinary water | | 440 |
| Boiled water | | 240 |
| $10^{-3}$ N KCN solution | Fertilized eggs | 200 |
| | Unfertilized eggs | 120 |
| Non-activated eggs from coelomic fluid | | 60 |

*Oxygen*. Oxygen diffusion through the egg membranes also plays some role in regulating the rate of membrane-hardening. The following experiments have been carried out. Inseminated salmon eggs were placed in boiled water under a layer of liquid paraffin. The eggs started to develop normally but membrane toughness increased more slowly than in control eggs (Table 8). Unfertilized and fertilized salmon eggs were also kept in a solution of $10^{-3}$ N KCN. The perivitelline space formed, but the toughness of the membranes increased at a slower rate than in control eggs (Table 8).

### 5. *The preparatory period of membrane-hardening*

Hardening of salmon, trout, and whitefish eggs does not begin immediately after fertilization but 60–180 minutes later (Text-fig. 2); whereas the hardening enzyme is only necessary within the first few minutes following fertilization or activation. Hence there exists a preparatory period of membrane-hardening when the effect of the enzyme has been completed and the preparatory processes in the membrane are in full swing though membrane toughness has not as yet increased but has even somewhat dropped. The elucidation of the nature of the processes occurring during this period seems to be very important for our inquiry into the mechanism of membrane-hardening.

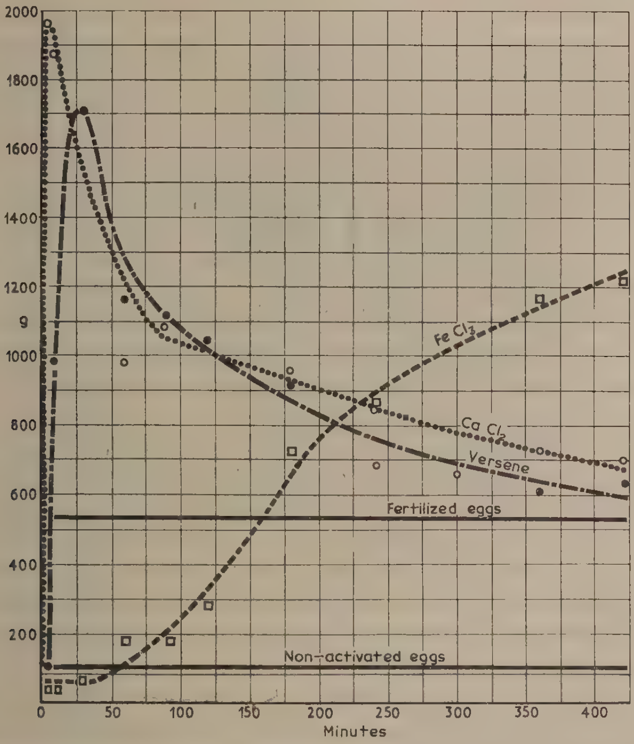
The salts of mono-, di, and trivalent metals have been shown above to exercise an appreciable effect upon membrane-hardening. The question arises whether this effect is specific for the preparatory period or is directly related to the increase in membrane toughness. To answer this question, salmon eggs were transferred 10, 30, 60, 80, 120, 240, and 360 minutes after fertilization from water to solutions of 0·1 N LiCl, $CaCl_2$, $FeCl_3$, and versene. It will appear from Text-fig. 9 that the effect of LiCl and $CaCl_2$ (inhibitory and stimulating) is particularly pronounced at the beginning of the preparatory period but gradually diminishes to disappear toward the end of this period. Hence the inhibitory or stimulating salt effect upon membrane-hardening is subsequent to the effect of the enzyme but precedes the commencement of the toughness increase.



Text-fig. 9. The effect of 0·1 N $CaCl_2$ and LiCl solutions on hardening of salmon egg membranes transferred to these solutions at certain time intervals (indicated by arrows) following fertilization. (Each point is the average of five measurements.)

The periodicity of the membrane-hardening processes becomes particularly apparent when 0·1 N $FeCl_3$ and versene are used. It will appear from Text-fig. 10 that versene exercises a quite different action upon various stages of the hardening processes. Within the first 5 minutes following fertilization 0·1 N versene completely inhibits hardening of the membranes. This is the period when the enzyme affects membrane-hardening, for which Ca ions are indispensable. During the preparatory period versene greatly accelerates these processes but as soon as this period is completed the effect of versene upon membrane-hardening gradually vanishes. 0·1 N $FeCl_3$ inhibits the hardening processes during the preparatory period. After completion of this period (beginning at 150 minutes after fertilization) the effect of $FeCl_3$ changes to the opposite.

During the preparatory hardening period LiCl, versene, $CaCl_2$, $MgCl_2$, $FeCl_3$, $AlCl_3$, and other salts call forth irreversible changes in the membranes which inhibit or stimulate the hardening process since only a short exposure of the membranes to these solutions seems to be necessary. Special experiments showed that 10 minutes after fertilization exposure of salmon eggs even for 1 minute to 0·1 N LiCl solution inhibits membrane-hardening, while 1-minute exposure to 0·1 N $CaCl_2$ solution accelerates the process, although a longer (10–30-minute) exposure enhances the stimulating or inhibitory action of these solutions.



TEXT-FIG. 10. Membrane toughness of salmon eggs 9 hours after transfer to 0·1 N $CaCl_2$, $FeCl_3$, and versene solution. Against the abscissa time is plotted from the moment of fertilization to transfer of eggs to the solutions.

It has been shown by Nakano (1956) in a study of the effect of various substances upon the membranes of fertilized *Oryzias* eggs that distilled water and solutions of cysteine and thioglycollic acid call forth a particularly pronounced swelling of the membranes provided the eggs are treated with these solutions during the first 10 minutes after fertilization. In his view, these data indicate the existence of a transitional period of egg membrane changes in *Oryzias* when the membranes reach their definitive structure. It would seem premature, however, to compare the transitional period of egg membrane changes in *Oryzias* with the preparatory period of membrane-hardening in salmonid eggs as described in the present paper.

## DISCUSSION

What is then the nature and mechanism of egg membrane-hardening? The solution of this problem is as yet difficult because we lack the necessary data concerning the accompanying chemical processes.[1] It may, however, be tentatively assumed that the increase in membrane toughness is due to the formation of some high polymer substances. It is generally held that the formation of many high polymer compounds is accomplished through a chain reaction and that the process of chain polymerization can be divided into the following stages: beginning of the chains, their growth, and their rupture (Semenov, 1936; Korshak, 1950). Presumably these three stages correspond to the above-mentioned three stages of membrane-hardening, viz., the preparatory latent hardening period, rapid increase in membrane toughness, and the cessation of increase in toughness (Text-figs. 2 and 3).

The chemists distinguish initiated and catalytic polymerization according to the mechanism of formation of the active molecule at the beginning of chain growth. The former occurs only in the presence of initiators (these are mostly various peroxides, diazo-compounds, and hexaaryl-ethanes) which are decomposed in the course of the reaction while their fragments are incorporated into the forming polymers. The latter reaction occurs only in the presence of catalysts which participate in the intermediary reactions but are not incorporated into the formed polymers (Korshak, 1950).

Since hardening of the egg membranes in salmonids is only possible in the presence of the hardening enzyme it might seem legitimate to assume that, if hardening is linked with polymerization of some substances, polymerization proceeds according to the catalytic type. However, it seems more probable that the process follows the type of initiated polymerization. This is suggested by the following considerations.

The beginning and growth of chains in catalytic polymerization is linked with the formation of active complexes between the molecule of the catalyst and the monomers. Hence the catalyst should take part in every act of new monomer addition and of chain growth. And yet the effect of the hardening enzyme was shown to be necessary only during the first few minutes following fertilization or activation after which hardening may progress without the enzyme. It would thus appear that the hardening enzyme is indispensable only at the very beginning of the process but takes no part in the subsequent links.

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[1] After the completion of this work I had an opportunity to read the very interesting works of T. S. Yamamoto (1956, 1957 *a*, *b*, *c*) and Hamano (1957), which together with the data obtained by Nakano (1956), give us some evidence of the chemical composition and structure of the egg membranes of teleosts, especially the membranes of salmonid eggs. The continuation of such work may in future make it possible to reveal the processes taking place during the hardening of fish egg-membranes at the chemical level.

If we accept the catalytic mechanism of polymerization underlying membrane-hardening it is hardly possible to account for the biphasic effect of salts. The drastic acceleration of hardening by divalent ions and versene during the latent period and abrupt inhibition of the process by 0·1 N LiCl, $AlCl_3$, and $FeCl_3$ during the same period might suggest that the respective chemical reactions are greatly different from those accomplished during the increase in membrane toughness.

All these peculiarities of the hardening process may be satisfactorily accounted for by the assumption that it belongs to the type of initiated chain polymerization. The hardening enzyme induces in the membranes the formation of initiators of polymerization, which is followed by an auto-catalytic process of increase in their amount. As soon as a certain concentration of the initiators is reached, chain polymerization in the membranes is elicited according to the conventional scheme: formation of active centres and beginning of chain growth, growth of chains and rupture of chains. $CaCl_2$, $MgCl_2$, and $SrCl_2$ accelerate the increase in the amount of the initiators while LiCl, $AlCl_3$, and $FeCl_3$ inactivate them. These salts do not affect the chain growth process itself while eventually producing an opposite effect: 0·1 N $FeCl_3$ accelerates growth of the chains although inhibiting it during the hardening period.

## SUMMARY

1. Unfertilized eggs of the trout (*Salmo trutta* m. *lacustris* Linn.) and of salmon (*S. salar* m. *sebago* Girard) can resist a pressure of 160–280 g. (toughness of the membrane is 1,500–2,000 g./cm.$^2$) and whitefish egg (*Coregonus lavaretus baeri* n. *swirensis* Pravdin) 40–50 g. Following fertilization or activation the membrane toughness at first decreases. During this period the trout and salmon eggs can resist a pressure of 60–210 g., and whitefish eggs 20 g. After 60–120 minutes the toughness of the trout and salmon membrane, and after 120–80 minutes that of the whitefish membrane, begins to rise at a high rate attaining its maximum after 3–7 days in the trout and salmon, and after 1–2 days in the whitefish. At this time trout and salmon eggs can resist a pressure as high as 2,500–3,500 g. (membrane toughness 19,000–21,000 g./cm.$^2$) and those of the whitefish 1,800–2,200 g.

2. Following fertilization or activation a special enzyme is released from the egg surface which calls forth hardening of the membranes. The release of the enzyme is blocked by 0·1 N LiCl, NaCl, $CaCl_2$, $MgCl_2$, $AlCl_3$, and $FeCl_3$, and is stimulated by water, in the medium. The release of the hardening enzyme

from the egg surface is independent of secretion of those substances which cause the formation of the perivitelline space.

3. The hardening enzyme is unstable, being inactivated by 5-minute heating at 30°. The effect of the enzyme upon the membranes is only necessary during the first 6–7 minutes following fertilization, whereupon hardening progresses equally well in its absence. Ca ions are necessary for the accomplishment of the enzyme action.

4. The course of further hardening after enzyme action can be divided into three stages, viz., a preparatory period when membrane toughness is somewhat decreased, a period of rapid increase in membrane toughness, and a period of maximum value with no further change. During the preparatory period 0·1 N LiCl, $AlCl_3$, and $FeCl_3$ produce an inhibitory effect while 0·1 N $CaCl_2$, $MgCl_2$, and $SrCl_2$ accelerates the hardening process. The passage of water across the membrane during the formation of the perivitelline space within the first 25–30 minutes after fertilization retards the hardening process. Oxygen deficiency likewise inhibits membrane-hardening.

5. The possible mechanism of membrane-hardening is discussed. It is suggested that membrane-hardening is associated with a process of initiated chain polymerization of substances within the membranes.

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# Genetical Studies on the Skeleton of the Mouse

## XXIV. Further Data on Skeletal Variation in Wild Populations[1]

*by* M. S. DEOL[2]

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A PILOT experiment by Weber (1950) established the fact that the minor skeletal variations universally present in strains of tame mice are also encountered in wild populations; and that the incidence of individual variants may differ widely from population to population. In the decade since Weber's work many new variants have come to light, and it seemed desirable to repeat his observations on the more extensive range of variants now available. An opportunity to do so presented itself in 1956 when wild mice from various localities in the eastern U.S.A. became available for study.

These animals had been collected for a totally different purpose. As is well known through the work of Dunn and his collaborators, there exists in the mouse a semi-dominant gene ($T$) for Brachyury or short-tail which in $T/+$ heterozygotes shortens the tail to a varying extent. In heterozygotes with a whole series of recessive alleles $t^0$, $t^1$, $t^2$, $t^3$, . . ., &c., a tailless phenotype is produced. As shown by Dunn and his colleagues (Dunn, 1956, 1957; Dunn & Suckling, 1956), these $t$ factors are widely spread in wild mouse populations. Over twenty populations from various parts of the U.S.A. have been tested and, with one exception, were proved to contain such factors. As the $t$ factors are recessive, their presence is discovered by a genetical test: wild mice are mated to Brachyuric ($T/+$) mice, and if the progeny includes tailless ($T/t^w$) individuals which, mated together, give rise to a balanced stock ($T/T$ and most $t^w/t^w$ homozygotes being inviable), the genotype of the tested mouse is considered to be established as $+/t^w$. As this kind of test is laborious and thus restricts the number of individuals which can be tested, it was hoped that the discovery of a suitable skeletal variant diagnostic for $t^w$ might simplify the procedure. This hope has been disappointed. However, the material thus collected has proved useful for population studies.

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[1] The material was collected at Nevis Biological Station, Irvington-on-Hudson, N.Y., under contract AT(30–1)–1804, U.S. Atomic Energy Commission; the analysis of the data was completed at the present laboratory.

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## MATERIAL

A total of 193 skeletons from four wild populations have been studied (Table 1); they were prepared by papain maceration. There were no $+/t^w$ mice in the Gull Island population; the other three included tested $+/t^w$ and $+/+$ mice, as well as some the genotype of which was not known on account of sterility, early death, &c. Most of the mice were actually caught in the wild, but some were bred in the laboratory from wild parents. The R–3 population (Levine & Dunn, 1956) was entirely laboratory bred and, as can be seen from Table 1, its origin was mixed. In addition to the papain preparations, twenty alizarin clearances of R–3 mice (10 $+/t^w$; 10 $+/+$) were also used, mainly to examine the bones and skeletal relationships which can be best studied *in situ*.

TABLE 1

*The origin of the four wild populations and the number of skeletons examined from each*

| Name | Origin | $t^w$ factor | Skeletons | | | |
|---|---|---|---|---|---|---|
| | | | $+/+$ | $+/t^w$ | ? | Total |
| G.I. | Great Gull Island, N.Y. | none | 30 | — | — | 30 |
| Vt. 1 | Norwich, Vt. | $t^{w14}$ | 23 | 1 | 12 | 36 |
| Ct. 2 | Storrs, Ct. | $t^{w11}$ | 15 | 11 | 25 | 51 |
| R–3 | N.Y. City ♂ × Philadelphia ♀ | $t^{w2}$ | 12 | 28 | 36 | 76 |

## RESULTS AND DISCUSSION

No obvious differences between the skeletons of $+/t^w$ and $+/+$ mice were discovered in either kind of preparation. While statistical differences between these two groups might be detectable in larger samples, they would be of little value in determining the genotype of a mouse with regard to $t^w$ factors.

The papain preparations were then classified for thirty minor variants which have been extensively used in the study of inbred strains (Deol, Grüneberg, Searle, & Truslove, 1957; Carpenter, Grüneberg, & Russell, 1957). However, not all of these were observed in the wild mice. The following variants were not encountered:

(1) Interfrontal-frontal fusion.
(2) Frontal fontanelle.
(3) Squamosal-parietal fusion.
(4) Foramen ovale open posteriorly.
(5) Periotic-occipital fusion.
(6) Inframaxillary crest.
(7) Interparietal-occipital fusion.
(8) Dyssymphysis of Thoracic I.

(9) Dystopia of processus spinosus of Thoracic II.
(10) Dyssymphysis ischio-pubica.

Most of the above variants have not yet been found outside the C57BL strain, in which they were originally discovered. Another variant, absence of lower third molar, is not mentioned in the tables as it occurred only once: a male in the G.I. population was affected on the right side.

TABLE 2

*Percentage incidence of twenty skeletal variants in four wild populations and four inbred strains*

| No. | Variant | *Wild populations* | | | | *Inbred strains* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *G.I.* | *Vt. 1* | *Ct. 2* | *R–3* | *CBA* | *A* | *C57BL* | *BALB/c* |
| 1 | Lacrimal-maxilla fusion . | 13·3 | 2·8 | 18·6 | 2·0 | 0·3 | 56·3 | 15·8 | 5·0 |
| 2 | Parted frontals . . . | 100·0 | 61·1 | 43·1 | 42·1 | 86·5 | 31·5 | 7·2 | 85·0 |
| 3 | Fused frontals . . . | 20·0 | 13·9 | 0·0 | 13·2 | 0·0 | 4·0 | 4·2 | 0·0 |
| 4 | Interfrontal . . . | 26·7 | 22·2 | 5·9 | 1·3 | 86·2 | 11·7 | 85·6 | 0·0 |
| 5 | Foramen ovale single . . | 40·0 | 26·4 | 28·4 | 14·5 | 62·3 | 44·7 | 3·0 | 90·0 |
| 6 | Alae palatinae* . . . | 0·0 | 26·4 | 14·7 | 42·8 | 4·4 | 0·0 | 72·1 | 0·0 |
| 7 | Presphenoid, preoptic sutures | 8·6 | 27·8 | 31·3 | 41·5 | 95·0 | 0·5 | 36·0 | 57·5 |
| 8 | Presphenoid, metoptic roots abnormal . . . | 6·9 | 11·1 | 4·9 | 25·0 | 72·5 | 22·5 | 1·1 | 97·5 |
| 9 | Foramen sphenoidale medium | 66·7 | 33·3 | 27·5 | 57·9 | 12·0 | 7·3 | 25·7 | 35·0 |
| 10 | Foramen hypoglossi single . | 48·3 | 62·5 | 56·9 | 62·5 | 85·4 | 31·6 | 75·2 | 42·5 |
| 11 | Processus pterygoideus. . | 3·3 | 16·6 | 3·9 | 1·9 | 0·3 | 0·0 | 20·7 | 2·5 |
| 12 | Accessory mental foramen . | 1·7 | 0·0 | 1·0 | 2·6 | 24·0 | 25·7 | 26·4 | 20·0 |
| 13 | Foramina transversaria imperfecta of C V . . | 1·7 | 0·0 | 0·0 | 19·1 | 0·0 | 0·3 | 66·2 | 0·0 |
| 14 | Tuberculum anterius inflexum | 3·3 | 0·0 | 0·0 | 0·7 | — | 11·9 | 3·0 | 17·5 |
| 15 | Cervical fusions . . . | 3·3 | 0·0 | 15·7 | 0·0 | 0·0 | 0·2 | 3·7 | 0·0 |
| 16 | Dyssymphysis of processus spinosus of Thoracic II . | 3·3 | 0·0 | 0·0 | 0·0 | 0·0 | 0·0 | 0·8 | 0·0 |
| 17 | Absence of tuberculum anterius of C VI . . . | 0·0 | 0·0 | 0·0 | 0·7 | 6·3 | 1·1 | 23·9 | 0·0 |
| 18 | Dyssymphysis of Thoracic X | 3·3 | 8·3 | 0·0 | 0·0 | — | — | 4·5 | 0·0 |
| 19 | Sacralization of L VI . . | 16·6 | 0·0 | 6·9 | 11·8 | — | 7·7 | 5·9 | 37·5 |

* This character has so far gone under the name of 'posterior border of the palatine'. It refers to a wing-like flap of bone which may be present on the postero-lateral border of the palatine. The new designation here proposed is both shorter and more descriptive.

The frequencies of various characters in the four populations are given in Table 2. In the case of bilateral characters, the percentage of affected sides rather than animals is taken. Table 3 gives the results of significance tests carried out to compare each of the four wild populations with the rest in turn. The $\chi^2$ method, with Yates's correction where necessary, was used for central characters; and Fisher's 'exact' method if the smallest class was 3 or less. Smith's method (Appendix to Grüneberg, 1955) was used for bilateral characters. In Table 3 a — sign indicates that there is no significant difference between the two values; + means a probability between 0·050 and 0·021; + + a probability between 0·020 and 0·011; + + + a probability between 0·010 and 0·001; and + + + + indicates a probability smaller than 0·001.

TABLE 3

*Results of significance tests carried out to compare each population with the rest in turn. (Explanation of symbols in the text)*

| | | *Populations compared* | | | | | |
|---|---|---|---|---|---|---|---|
| *No.* | *Variant* | *G.I. and Vt. 1* | *G.I. and Ct. 2* | *G.I. and R–3* | *Vt. 1 and Ct. 2* | *Vt. 1 and R–3* | *Ct. 2 and R–3* |
| 1 | Lacrimal-maxilla fusion | + | — | +++ | ++++ | — | ++++ |
| 2 | Parted frontals | ++++ | ++++ | ++++ | — | — | — |
| 3 | Fused frontals | — | +++ | — | +++ | — | +++ |
| 4 | Interfrontal | — | ++ | ++++ | + | ++++ | — |
| 5 | Foramen ovale single | — | — | ++++ | — | — | — |
| 6 | Alae palatinae | ++++ | ++++ | ++++ | — | — | ++++ |
| 7 | Presphenoid, preoptic sutures | ++ | +++ | ++++ | — | — | — |
| 8 | Presphenoid, metoptic roots abnormal | — | — | +++ | — | — | +++ |
| 9 | Foramen sphenoidale medium | +++ | ++++ | — | — | ++ | ++++ |
| 10 | Foramen hypoglossi single | — | — | — | — | — | — |
| 11 | Processus pterygoideus | ++ | — | — | +++ | ++++ | — |
| 12 | Accessory mental foramen | — | — | — | — | — | — |
| 13 | Foramina transversaria imperfecta of C V | — | — | ++++ | — | ++++ | ++++ |
| 14 | Tuberculum anterius inflexum | — | — | — | — | — | — |
| 15 | Cervical fusions | — | — | — | ++ | — | ++++ |
| 16 | Dyssymphysis of processus spinosus of Thoracic II | — | — | — | — | — | — |
| 17 | Absence of tuberculum anterius, C VI | — | — | — | — | — | — |
| 18 | Dyssymphysis of Thoracic X | — | — | — | — | + | — |
| 19 | Sacralization of L VI | ++++ | — | — | + | ++++ | — |

The data in Table 3 show that in 80 of the 120 possible comparisons, there were no significant differences. Nine differences of low significance (+ and + +) are probably largely due to accidents of sampling and may be ignored. On the other hand, few, if any, of the thirty-one highly significant differences (+ + + and + + + +) can be ascribed to chance alone. Each one of the four wild populations differs from the rest in the incidence of at least three variants; the average number of highly significant differences between any two populations being five. In turn, 13 out of 20 variants encountered show highly significant inter-population differences (all the 20 variants have previously been shown to be, at least partly, under genetic control).

The significant inter-population differences can be expressed in terms of the standard deviation by means of probit transformations. Populations in which a variant was absent have for this purpose been treated as if it had been present in one-half of an individual; and similarly one population with a 100 per cent. incidence has been treated as if one-half of a mouse had failed to show the character. The average of the forty significant differences amounts to 1·14 standard deviations.

The range of percentages of the skeletal variants in the four wild populations is by and large comparable with that in inbred strains of mice (see Table 2; for additional data see Deol, Grüneberg, Searle, & Truslove, 1957; Carpenter, Grüneberg, & Russell, 1957). One exception seems to be the presence of accessory mental foramina. These are uncommon in all four wild populations but very frequent in nearly all tame mice examined; of the 11 inbred strains and sub-strains examined, 9 varied between 20 and 64 per cent., while only 2 showed an incidence of 8 per cent.; an $F_1$ generation between two inbred strains had 43 per cent. accessory mental foramina. The large difference between wild and tame mice in this respect can hardly be due to chance alone; on the other hand, it is not easy to see why this seemingly trivial variant should be so rare in wild mice.

The foramina transversaria imperfecta are common in the R–3 population (it occurs 7 times on the right, 12 times on the left, and 5 times bilaterally in 76 mice) but do not involve the C VI vertebra. In this respect R–3 differs from various European populations (Weber, 1950), and the C57BL inbred strain (Grüneberg, 1950*a*) in which the C V and C VI vertebrae are about equally affected (C VI is affected more intensely than C V in the presence of the undulated gene; see Grüneberg, 1950*b*).

The variants used in this investigation are partly under genetic control. This is shown by the large inter-strain differences under constant laboratory conditions. On the other hand, it is known that deficient diets can influence the incidence of most of these entities to a considerable extent (Searle, 1954; Deol & Truslove, 1957). As the wild populations have lived under different environmental conditions, it is probable that both sources of variation have contributed towards the inter-population differences. The genetical component could be determined by breeding wild mice for a minimum of two generations under constant laboratory conditions; as the present observations were coincidental to a larger research programme, this was not practicable. However, judging from the general nature of the effects on the skeleton produced by deficient diets, I would regard it as improbable that the inter-population differences reported here are mainly due to differences in the environment. If this is accepted, the question arises of whether the genetical inter-population differences are adaptive and hence the result of natural selection, or whether they are due to genetic drift (the Sewall Wright effect) and hence purely accidental. The solution of this important question would require elaborate experiments both in the field and in the laboratory.

## SUMMARY

Four populations of wild mice (*Mus musculus* L.) derived from different parts of the eastern U.S.A. have been classified for an array of some twenty minor skeletal variants which, from other work, are known to be partly under genetic control. The four populations differed from each other highly significantly in the incidence of, on an average, five of these variants. It is not known whether the genetical component of the inter-population variance is adaptive in nature, or whether it has originated by genetic drift.

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# The Development of the Adrenals and Sympathetic Paraganglia in certain Madagascar Insectivores

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WITH THREE PLATES

THE development of the adrenal gland in the insectivores has not been extensively studied, Aichel (1900) and Soulié (1903) having described it in the mole (*Talpa*) and Celestino da Costa (1920, 1926) in the hedgehog (*Erinaceus europaeus*). Both Soulié and Celestino da Costa agree that in these animals the adrenal develops basically much as in other mammals, i.e. the cortical primordium comes from the coelomic epithelium, and is later invaded by migrating elements (phaeochromoblasts) from the adjacent sympathetic anlage (Aichel, however, regards the two as having a common origin, from remains of nephrostomal canals of the upper part of the mesonephros).

Nevertheless, there are some notable differences in time relations, at least in the hedgehog, for the invasion of the cortical primordium by medullary elements occurs unusually early (8 mm.): 'La pénétration débute assez précocement à une époque où il est encore douteux qu'il existe deux espèces cellulaires dans l'ébauche proto-sympathique' (Celestino da Costa, 1926, p. 157) (it appears to be later—about 20 mm.—in *Talpa*, according to Soulié, 1903, p. 515). Moreover, these cells differentiate rapidly within the adrenal into paraganglionic (medullary) cells, and, although the immigration of new cells from the sympathetic may continue and the adrenal gland may not attain its definitive form for some time, yet it is highly organized as far as the medulla is concerned well before birth (27 mm.), much sooner than is the case in other mammals (Celestino da Costa, 1926, p. 157).

There is another point of contrast, as Celestino da Costa points out, between the development of paraganglionic tissue in the hedgehog and that in other mammals. As is well known, in most mammals sympathetic elements, comparable to those which invade the adrenal, mass themselves also in front of the aorta in this region; here they differentiate either into sympathetic ganglion cells of the pre-aortic ganglia, or into cells which collectively form the pre- (or juxta-) aortic paraganglia (organ of Zuckerkandl, 1901), and which, in contrast to the adrenal

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medulla (medullary paraganglion) are usually well developed at birth. In the hedgehog, however, it is different—in fact almost the reverse—for, although paraganglionic cells (phaeochromoblasts) differentiate quickly *within* the adrenal, those outside it 'remain for a long time in a rudimentary condition' (Celestino da Costa, 1935*b*, p. 480), and probably do not begin to differentiate until after birth. Whereas in most other mammals there is a fairly rapid post-natal regression, in the hedgehog these extra-adrenal paraganglia are apparently well developed even in the adult (Celestino da Costa, 1935*b*, p. 480).

In addition to the abdominal paraganglionic tissue, Celestino da Costa (1936*c*, p. 99) found a small mass of cells in the cervical sympathetic of a hedgehog embryo (age not stated) 'possédant les caractères de vrais éléments paraganglionnaires'. This finding is of particular interest for it calls attention to the potentiality of any part, or all parts of the sympathetic to form paraganglionic tissue, although generally this is not obtrusive in most mammals (according to Truex, 1950, it is very exceptional to find such cells *within* sympathetic ganglia, although they may occasionally be seen alongside them). But in the Vespertilionidae this capacity of the sympathetic to form paraganglionic tissue ubiquitously is strikingly evident, for in the embryo such tissue accompanies the greater part of the sympathetic chain, although it later regresses in part (Celestino da Costa, 1917, 1923, 1935*b*, 1936 *a*, *b*, *d*, *e*, 1940; Stark, 1955).

These facts have an important bearing on the present study. While studying the development of the vessels in the region of the carotid fork in insectivore embryos of the Bluntschli Collection in this Department, I noticed in an *Ericulus* embryo a peculiar structure at the bifurcation, which at first sight looked like an aberrant nodule of adrenal cortical tissue (the possibility that it might have been a peculiar type of parathyroid III, still located at the carotid fork, was readily excluded by the difference in structure between it and parathyroid IV, which is embedded in the thyroid). This nodule proved to be part of a continuous column throughout the neck and thorax, merging eventually with the adrenal gland. A closer study of the development of this tissue in three different species of insectivores indicates, however, that it is not cortical tissue at all but rather an extensive column of precociously developed paraganglionic tissue, lying in close association with the sympathetic chain, such as is also found in the Vespertilionidae.

## MATERIAL AND METHODS

With the exception of a block of tissue from a late embryo of *Centetes*,[1] which was sectioned and stained specially with chrome alum-haematoxylin for cytological purposes, all the material used in this study had been previously prepared by Professor Bluntschli. This comprised various stages in the development of *Centetes ecaudatus*, *Hemicentetes semispinosus*, and *Ericulus setosus*. No one series was sufficiently complete to give a satisfactory picture of the entire development, but the similarities in the development of the three species were sufficiently close to enable a composite picture to be drawn of the development in the Tenrecidae as a whole.

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[1] For convenience I have retained the generic names as used by Prof. Bluntschli, instead of those given by Simpson (1945), i.e. *Centetes* instead of *Tenrec*, and *Ericulus* instead of *Setifer*.

Seven embryos of *Ericulus* showed various stages of adrenal development; these ranged from 27 somites to 26-mm. crown–rump length (placental stages—Strauss, 1943—from $F_1$ to L). Nine *Centetes* embryos were available, of which only one (6 mm. greatest length) was early; the rest ranged from 20 mm. greatest length to 72 mm. (about term). The 10 available *Hemicentetes* embryos were all young, from 26 somites to 13 mm. (placental stages—Goetz, 1937—from IX to XIII). The embryos had been fixed either in formol or Susa, and with one exception had been embedded in paraffin. Except for 4 series, they were sectioned at 10 μ, and the sections were variously stained with haematoxylin-eosin, iron haematoxylin-orange G, or azan (see Table 1).

TABLE 1

| *No.* | *Insectivore series* | *Size* | *Fixation* | *Thickness* | *Staining* |
|---|---|---|---|---|---|
| *Ericulus setosus* | | | | (μ) | |
| E 1 | 51 | 27 somites | 8% formol; Susa | 10 | hx-eosin; iron hx-orange G |
| E 2 | 51*a* | 33 somites | 8% formol | 10 | hx-eosin; iron hx-orange G |
| E 3 | 56 | 29 somites | 8% formol | 10 | iron hx-orange G |
| E 4 | 74 | *c.* 12 mm. | 7% formol | 10 | azan; iron hx-orange G |
| E 5 | 57 | 15 mm. C–R | 8% formol | 10 | azan |
| E 6 | 55 | 16 mm. C–R | 8% formol | 10 | azan |
| E 7 | 58 | 26 mm. C–R | Susa | | |
| E 7*a* | (head) | | | 10 | azan |
| E 7*b* | *z* (trunk) | | | 10 | azan; iron hx-orange G |
| *Centetes ecaudatus* | | | | | |
| C 1 | 50*a* | *c.* 6 mm. | 8% formol | 10 | iron hx-orange G |
| C 2 | 32 | *c.* 20 mm. | Susa | 20 | azan |
| C 3 | 33 | 20 mm. | Susa (decalc.) | 10 | azan |
| C 4 | 20 | 21 mm. | Susa (decalc.) | 10 | hx-eosin; azan |
| C 5 | 19 | 22 mm. | Susa (decalc.) | 10 | hx-eosin; azan |
| C 6 | 2 | 23·5 mm. | Susa | | |
| | *a* (head) | | | 15 | hx-eosin; azan |
| | *b* (trunk) | | | 10 | hx-eosin; azan |
| C 7 | 3 | 23 mm. | Susa (decalc.) | 15 | hx-eosin; azan |
| C 8 | 66*a* | 69 mm. | formol | 7; 10 | chrome alum-hx; hx-eosin |
| C 9 | 65 | 72 mm. | formol | 15 | hx-eosin; azan |
| *Hemicentetes semispinosus* | | | | | |
| H 1 | 38*b* | 26 somites | Susa | 10 | hx-eosin; iron hx-orange G; azan |
| H 2 | 48 | 32 somites | ,, | 10 | iron hx-orange G |
| H 3 | 14*a* | *c.* 4·75 mm. | ,, | 10 | hx-eosin; azan |
| H 4 | 39 | 40 somites | ,, | 10 | hx-eosin; iron hx-orange G; azan |
| H 5 | 18 | 8·5 mm. | ,, | 10 | hx-eosin; azan |
| H 6 | 11 | *c.* 8·5 mm. | ,, | 10 | hx-eosin; azan |
| H 7 | 17 | *c.* 8·5 mm. | ,, | 10 | hx-eosin; azan |
| H 8 | 46 | 12·5 mm. | ,, | 10 | hx-eosin; iron hx-orange G |
| H 9 | 12 | 13 mm. | ,, | 10 | azan |
| H 10 | 1 | 13 mm. | ,, | 10 | hx-eosin; azan |

## RESULTS

Three embryos were available at an age when the first signs of adrenal cortex formation could be seen.

In a 33-somite *Ericulus* embryo (E 2) proliferation of coelomic epithelium can be seen medial to the mesonephros, and adjacent to the aorta and the root of the mesentery (Plate 1, fig. 1). There is no sign of gonadal proliferation, and the mesonephros is still rudimentary. The sympathetic primordium (primary sympathetic; protosympathetic of Celestino da Costa) is present as a well-defined but discontinuous column of comparatively large cells, dorsolateral to the aorta; it appears to be quite independent, without fibrous or cellular continuity with the neural tube or neural crest.

In *Hemicentetes* embryos of about the same age, the cortical primordium is less distinct. At a stage (H 1) when the sympathetic anlage is just appearing but there is no gonadal proliferation, cords of cells extend in from the coelomic epithelium in the same situation as the proliferation in *Ericulus* (Plate 1, fig. 2). A little later (H 2), gonadal proliferation has begun, but, owing to twisting of the embryo and the consequently awkward plane of section, it was difficult to distinguish the cortical primordium from the rostral end of the gonad; certainly, the adrenal primordium, if present, is quite continuous with the developing gonad. By 4·75 mm. (H 3), the cortical primordium is a compact mass of cells not yet divorced from the coelomic epithelium, but already well vascularized. Medial to it is the aorta, and just laterally the mesonephric corpuscles. Its relation to the developing gonad is particularly intimate; in fact, the two appear to be quite continuous (Plate 1, fig. 3). The protosympathetic is now a distinct column of largish cells, in active mitosis, dorsolateral to the aorta. Ventral migration of these cells is just starting, but there is no invasion of the cortical primordium. However, in a slightly older embryo (H 4), ventral migration is well under way (Plate 1, fig. 4) and paraganglion cells lie not only ventral to the aorta, but also in relationship with the cortical primordium, which is still intimately associated with the gonad (Plate 1, fig. 5).

The youngest available, but slightly older, embryo of *Centetes* (C 1) is somewhat similar. Here, however, the cortical primordium is separate from the coelomic epithelium but only partially encapsulated; cells are still coming from the coelomic epithelium, but the relationship with the gonad is now less intimate (Plate 1, fig. 6), although the two are still continuous in part. Ventral migration of cells from the sympathetic is now extensive; these cells lie both adjacent to and within the cortical primordium (Plate 1, fig. 6), and even reach the dorsal aspect of the gonad. Below this level, these cells form a mass on each side, connected caudally by a bridge of similar cells. The rostral part of the mesonephros, now dwindling, is at some distance from the adrenal; the metanephros is still rudimentary, and caudally placed. The sympathetic itself, in contrast to the elements which have migrated ventrally from it, is a continuous column of small undifferentiated cells with hyperchromatic nuclei, and rami communicantes are present; but, occasionally, it presents nests of larger paraganglionic cells.

As development proceeds, although there is little differentiation of sympathoblasts, development of the paraganglionic tissue is rapid, particularly in the adrenal region. Thus in *Hemicentetes*, by 8·5 mm. (H 5, 6, 7), the pre-aortic paraganglionic tissue is a large well-vascularized mass prolonged rostrally on either side in the form of a U (Plate 2, figs. 7, 8). The upper lateral poles of this mass (which although little further differentiated is readily distinguishable from the sympathetic tissue proper) will form the adrenal medulla; here, paraganglion cells are in contact with the cortical primordium, and extensive invasion is occurring, particularly ventromedially (Plate 2, fig. 8). Both the paraganglionic tissue and the cortical primordium show active mitosis (Plate 2, fig. 9) and the latter, a compact mass of cells, is separate from the coelomic epithelium, but still in cellular continuity with it (Plate 2, fig. 9); it also retains its connexion with the gonad (Plate 2, fig. 10). In addition to those in the adrenal region of *Hemicentetes*, paraganglion cells occur sparsely in the thoracic sympathetic; they are distinctly larger than sympathoblasts, and have much paler nuclei. But their occurrence in *Hemicentetes* is negligible compared with that in *Ericulus* and *Centetes*; if extensive paraganglion development ever occurs here in conjunction with the sympathetic, it must be very evanescent.

Succeeding stages of development show still further growth and differentiation of paraganglionic tissue both inside and outside the adrenal (in *Ericulus* and *Centetes*, also in the cervical and thoracic regions). The cortical tissue, however, remains quiescent and is relegated to a crescentic cap of small cells over the rostral pole of the (medullary) paraganglion; this in turn is widely continuous with the rest of the pre-aortic paraganglionic tissue, which in *Centetes* and *Hemicentetes* forms a distinctive median pre-aortic mass.

Thus, in *Hemicentetes* by 12–13 mm. (H 8, 9, 10) the paraganglionic tissue forms a huge mass in front of the aorta, which extends rostrally on each side to come into relationship with the investing cap of cortical cells (Plate 2, fig. 11). The paraganglion cells, both inside and outside the adrenal, now have a clearly epithelioid appearance, with increased cytoplasm and vesicular nucleus; they still show mitoses and form wavy irregular cords, two or three cells thick, with wide sinusoidal capillaries intervening. Over the rostral poles of this mass, on either side, the cortical cells form a thin covering to the medullary paraganglion; they are small cells with dark nuclei, and still comparatively undifferentiated. Deeply, they project into the medulla in quite an irregular fashion, forming a 'boundary zone' in which there is considerable intermingling of the two tissues. At this stage, too, we find secondary invasion of the medullary (and to some extent extra-medullary) paraganglion, by sympathoblasts migrating ventrally from the sympathetic chain, where differentiation of ganglion cells is still proceeding slowly. As before, very little paraganglionic tissue occurs in conjunction with the sympathetic chain in *Hemicentetes*; it is found occasionally in the thoracic sympathetic.

In *Ericulus* embryos about the same age (15–16 mm.—E 4, 5, 6) development, both of paraganglionic and cortical tissue is much the same; the cells are well differentiated and the upper pole of each mass is capped by a thin cortex of small dividing cells (Plate 2, fig. 12). However, here the paraganglia on each side, although they come very close to each other, do not fuse to form a median mass; but the absence of a median pre-aortic paraganglion is compensated for by great development of paraganglionic tissue elsewhere, for it occurs throughout almost the entire length of the body, beside the sympathetic. Thus, there are two dorsal para-aortic columns of paraganglionic tissue, which, although varying in thickness, continue through the abdominal, thoracic, and cervical regions (Plate 3, figs. 13, 14, 16, 17) to the base of the skull, where they are in relation with the upper pole of the superior cervical ganglion (Plate 3, fig. 13). Caudally, they reach down to the future pelvic region, beyond the caudal bifurcation of the aorta. In the region of the developing adrenal where the paraganglion columns lie dorsal to the gland (Plate 3, fig. 14), they are freely continuous with the medullary paraganglion. The cells of these two columns, although more compactly arranged, are the same as those of the adrenal medulla—they are distinctly epithelioid in appearance, with eosinophil cytoplasm and vesicular hypochromatic nuclei. They form irregular cords, with an intervening network of sinusoidal capillaries, thus appearing very vascular (Plate 3, figs. 13, 14, 16, 17).

No *Centetes* embryos at this stage were available, but from a study of older embryos, the development is clearly similar, except that here there is a median pre-aortic paraganglion as in *Hemicentetes* (although not so large). The development of juxta-sympathetic paraganglionic tissue is more pronounced than in *Hemicentetes*, although not as great as in *Ericulus*, for the two para-aortic paraganglionic columns are not continuous but are broken into independent masses, associated with the ganglia of the cervical, thoracic, and abdominal sympathetic chain.

The subsequent development of the adrenal involves gradual elaboration of the cortex, and further differentiation of the paraganglionic tissue, particularly in its medullary part; elsewhere there may be slight regression (no embryos of *Hemicentetes* were available at these later stages).

In *Centetes*, by 20–23 mm. (C 2–7), the large adrenals consist almost entirely of paraganglionic tissue, capped dorsally and rostrally by a crescentic cortex, still comparatively thin, but now beginning to proliferate and differentiate. The small cells with hyperchromatic nuclei which in *Ericulus* and *Hemicentetes* previously made up the entire cortex, here form a thin superficial layer just under the cellular capsule; but in addition, there is now a deeper layer of rather larger, more epithelioid cells intermingling with those of the adjacent medulla. Thus the cortex now has two zones, although it is still quite thin. The paraganglionic tissue has also begun to show changes, particularly in the adrenal region. Instead of the previously compact cords of epithelioid cells, it is now of much looser texture, with more interstitial tissue. The cells tend to separate from each other, although still connected by numerous processes and they also have a more fusiform shape (this is less evident, however, in the juxta-sympathetic paraganglia). The abdominal paraganglionic mass, particularly its intra-medullary part, continues to be invaded by small sympathoblasts with dark nuclei, which form scattered nests along the nerves which have also penetrated the paraganglionic tissue. Like *Ericulus*, *Centetes* shows much paraganglionic tissue in a para-aortic position, beside the sympathetic chain; this is not a continuous column as in *Ericulus* but comprises separate paraganglia along the chain in the cervical (Plate 3, fig. 18), thoracic, and abdominal regions.

In *Ericulus* about this stage (26 mm.—E 7) there is also differentiation of the cortex, although less than in *Centetes*. The thicker cortex still consists of small cells projecting irregularly into the adjacent medulla; the deeper cells are only slightly larger than the superficial (Plate 3, figs. 20, 21). Furthermore, although the interstitial tissue is greatly increased, the paraganglion cells still form compact cords—the loosening and separation seen in *Centetes* has not yet begun here (Plate 3, figs. 20, 21). The distribution and structure of the extra-medullary paraganglionic tissue is much as it was earlier, i.e. it extends as a para-aortic column beside the sympathetic through the thorax (Plate 3, fig. 15), and into the neck up to the carotid fork (Plate 3, fig. 19) where, as in *Centetes* (Plate 3, fig. 18), it obviously has quite a different structure from the carotid body. Downwards, it reaches to the pelvic region. Although it may now be interrupted in part, it is still very abundant.

The features just described apparently persist during the rest of pregnancy. There is a continued gradual growth and differentiation, particularly of the cortex, but also of the paraganglionic tissue (especially in the adrenal region). Thus, in late *Centetes* embryos (C 8, 9) just before birth, the cortex is much thicker and forms a substantial cap to the medulla, which, however, remains quite continuous with the extra-adrenal paraganglionic tissue. The surface cortical cells still have round hyperchromatic nuclei and a high nucleo-plasmatic ratio; but the larger deeper cells are clearly epithelioid, forming anastomosing cords which interdigitate with those of the underlying medulla. There is thus a distinct 'zoning' of the cortex—the small superficial cells representing a 'glomerular' zone and the deeper cells a 'fasciculo-reticular' zone (Plate 3, fig. 22). The paraganglionic tissue (particularly of the adrenal medulla) is now much looser. The medullary cells have a more irregular form; their arrangement in cords is lost, and there is much intervening interstitial tissue, with sinusoidal capillaries. Nests of undifferentiated sympathoblasts are still numerous, scattered throughout the paraganglionic tissue in relation with nerve-trunks; but no differentiation of these cells had occurred, and their fate could not be determined. Elsewhere, the paraganglionic tissue, which is still well developed in these late embryos, tends to preserve its epithelial form and compact nature, and can still be traced well into the cervical region.

## DISCUSSION

Nothing in the development of the Tenrecidae suggests that the development of the adrenal gland does not proceed fundamentally as it does in other mammals, i.e. there is (*a*) *a cortical primordium* which develops (initially, probably just before the gonad appears) from the coelomic epithelium on each side of the mesenteric root, and medial to the mesonephros, while this is still rudimentary; and (*b*) *a medullary primordium* which, like the extra-medullary paraganglia, derives from the primary paravertebral sympathetic anlage (which according to Bruni, 1917, and Goormaghtigh, 1921, may be entirely paraganglionic), and comes into conjunction secondarily with the developing cortex.

The Tenrecidae show peculiarities which distinguish them, in respect of the development of the adrenal and paraganglionic tissue as a whole, from all other mammals except the Vespertilionidae. For in them the appearance and differentiation of the paraganglionic tissue is not only precocious but, in the case of *Ericulus* and *Centetes*, is much more abundant and extensive than usual. Thus the migration of phaeochromoblasts from the protosympathetic almost coincides with the appearance of the cortical primordium, and the two come together even before the cortical primordium has properly separated from the coelomic epithelium.

As for the development of the cortical primordium, which Soulié and Celestino da Costa derive from the coelomic epithelium, but Aichel from vestigial nephrostomal canals, the indications here support the former conclusion. Yet it seems that the proliferation may not at first be diffuse, for in *Hemicentetes* (and Soulié describes something similar in *Talpa*) cellular cords extend from the coelomic epithelium into the subjacent mesoderm. Although there is no superficial depression of the coelomic epithelium here, such as Aichel figured in the rat (and which he took to be vestigial nephrostomes), the appearances otherwise quite resemble those he shows (1900, Plate 1, fig. 2). But, as Soulié says (1903, p. 637), that need not mean that these epithelial cords are mesonephric canals.

The cortical primordium proliferates rapidly at first, and is soon vascularized. From the beginning, as with *Talpa* (Soulié) and *Erinaceus* (Celestino da Costa, 1920, 1926), and some other mammals (Goormaghtigh, 1921), it is contiguous with the developing gonad, from which it separates only later. Also, as it grows, it soon makes contact with mesonephric corpuscles and tubules, although here the parietal epithelium of the corpuscles did not appear to contribute to the cortical primordium, as Crowder (1957) says it may do in man.

The ventral migration of sympathetic elements which produces the medulla is only part of a more extensive process whereby phaeochromoblasts become massed in front of the aorta to form unusually large juxta-aortic paraganglia which in *Centetes* and *Hemicentetes* fuse to form a median pre-aortic mass. This paraganglion (of the bilateral masses in *Ericulus*) is perfectly continuous with that part which, superiorly and laterally, adjoins the developing cortical primordium, and which properly forms the primordium of the adrenal medulla. The cells forming this extensive paraganglion complex are at first small and basophilic with hyperchromatic nuclei; but they very rapidly enlarge—the cytoplasm increases and becomes eosinophilic, while the nucleus takes on a hypochromatic appearance. The tissue becomes highly vascular and the cells—now distinctly epithelioid—are compactly arranged in reticulated cords of markedly glandular appearance. Meanwhile, at the upper poles of this U-shaped mass, where the future adrenal will be, the cortical cells form a comparatively thin cellular cap over the paraganglionic tissue, to some extent invading it. These cortical cells remain undifferentiated—small, with hypochromatic nuclei—for some time.

So, even before mid-term, the adrenals in these insectivores consist almost entirely of a highly differentiated medulla, only partially covered by a thin undifferentiated cortex, incomplete infero-medially where the medulla remains widely continuous with the extra-medullary paraganglionic tissue, whether in the form of bilateral masses (*Ericulus*) or a large fused mass in front of the aorta (*Centetes*, *Hemicentetes*). This continuity between the medulla and the so-called 'free' paraganglionic tissue outside the adrenal is, of course, not peculiar to insectivores; Kohn (1903) and Wrete (1927) realized that it occurs commonly, and this has led Iwanow (1932) to suggest that the adrenal medulla is formed, not only by direct immigration of sympathetic elements, but also by inclusion and transformation of phaeochrome cells of the cranial part of the 'paraganglion aorticum lumbale' (organ of Zuckerkandl). But here the connexion between the two seems to be much more extensive; and, what is more, the intra-medullary paraganglionic tissue is differentated very early. At a time when, in man, it appears only as scattered islets of relatively undifferentiated tissue, in the insectivores it is well differentiated, aggregated, and forms the bulk of the adrenal gland.

In the latter half of pregnancy, both the cortical and medullary tissue continue to differentiate. In the case of the medulla (and the pre-aortic paraganglion), the epithelioid appearance is gradually lost and the cells tend to become more fusiform; the arrangement in cords is replaced by a more diffuse distribution of the cells; moreover, there is a secondary invasion by sympathoblasts, forming islands of small dark cells in relation with nerve-trunks, which may contribute to the medulla later. The cortex, too, shows differentiation, with gradual thickening; but it still forms a cap partially covering the medullary tissue which still remains freely continuous with the extra-medullary tissue—in fact it is difficult to decide how much of the paraganglionic tissue is truly adrenal. Furthermore—but as a very late development, just before birth—the cortex shows a zoning: superficially the cells are small, forming a 'glomerulosa', while deeply they enlarge to form cords of epithelioid cells which may be heavily vacuolated ('fasciculata' or 'fasciculo-reticularis'). This is the structure of the gland at birth; it certainly has not yet attained the degree of specialization seen in adult insectivores (Colson, 1910; Kolmer, 1918; Bourne, 1949).

In mammals generally it seems that, even if the intrusion of phaeochrome elements does occur early (Crowder, 1957), their differentiation is comparatively slow, and the medulla is not fully constituted until after birth (Hett, 1925); yet the pre-aortic paraganglia are well developed before birth (Hammar, 1934). In the hedgehog, however, Celestino da Costa has pointed out (1920, 1926) that the first invasion of sympathetic (phaeochrome) elements is always early, and their differentiation within the adrenal also starts soon (although it is prolonged); but here there is no comparable differentiation of paraganglionic tissue outside the adrenal, at least until after birth. Thus there is a marked difference in this respect between the Tenrecidae and Vespertilionidae in which it differentiates precociously in both situations, and the hedgehog on the one hand, and most mammals on the other.

In addition to the changes in the adrenal region, there is, in *Ericulus* and *Centetes*, an unusual development of paraganglionic tissue on a more dorsal plane, in conjunction with the sympathetic chain. This tissue—comparable in every way with the intra- and extra-adrenal paraganglionic tissue—forms a continuous (*Ericulus*) or discontinuous (*Centetes*) column throughout the abdomen, thorax, and neck. In young *Ericulus* embryos, it may reach the base of the skull, although later, both in *Ericulus* and *Centetes*, it extends only to the carotid fork; but throughout embryonic life, there is only slight regression of this paraganglionic tissue. In *Hemicentetes* this development is negligible, but, because occasional paraganglion nodules may be found in thoracic sympathetic ganglia, it is possible that it may appear at a certain stage of development but be more evanescent. At any rate, no extensive column of juxta-sympathetic paraganglia was seen in *Hemicentetes* embryos, although the pre-aortic paraganglionic tissue is remarkably well developed in this species.

Such a massive and extensive development of paraganglionic tissue in conjunction with the sympathetic chain, has, as far as I am aware, been described previously only in the Vespertilionidae (Schultze, 1897; Celestino da Costa, 1917, 1923, 1935*a*, 1936 *a*, *d*, *e*, 1940). The presence even of a few paraganglion cells in isolated parts of the chain otherwise appears to be exceptional (Truex, 1950) even in the hedgehog (although here the very large ganglia may contain a complement of undifferentiated paraganglionic cells, which could remain a long time dormant as 'metaneurogonia'—Celestino da Costa, 1926, 1935 *a*, *b*, 1954—and possibly, like the organ of Zuckerkandl, only differentiate after birth; yet I have never seen paraganglion cells in relation with the superior cervical ganglion in the adult hedgehog, as they are so obviously in *Ericulus* and *Centetes*).

The structure of this paraganglionic tissue at the bifurcation precludes the possibility that in the Tenrecidae the carotid body is derived from the same source. Celestino da Costa remarks of the Vespertilionidae 'which possess such a considerable paraganglionic system', that 'the carotid body has apparently nothing to do with these paraganglia. The cells forming the body are quite rudimentary elements that appear as the result of a cellular condensation in the wall of the carotid. Its structure does not support a common origin with the paraganglionic tissues' (Celestino da Costa, 1935*b*, pp. 482–3). This statement applies equally well to the Tenrecidae.

## SUMMARY

In the Tenrecidae, the adrenal cortex develops from the coelomic epithelium close by the developing gonad, with which it is for a time in contact. The medullary (paraganglionic) tissue arises precociously from the protosympathetic, invades the cortical primordium very early, and is freely continuous with the large pre-aortic paraganglia, which may be fused across the aorta (*Centetes*, *Hemicentetes*). Whereas the paraganglionic tissue (medullary and extra-medullary) differentiates rapidly, the cortex remains quiescent until just before birth, forming a cap of small cells over the paraganglionic tissue. Contributions from the sympathetic continue, but the fate of these small cells was not determined.

Furthermore, as in certain bats, so in *Centetes* and *Ericulus* the paraganglionic tissue is not confined to the adrenal region, but extends alongside the sympathetic chain as a distinctive continuous (*Ericulus*) or discontinuous (*Centetes*) column, the cells of which are as well-differentiated as in the abdominal paraganglia. In *Ericulus* this column reaches right to the base of the skull, and sections through the carotid fork show that the structure of the paraganglionic tissue is quite unlike that of the carotid body.

## ACKNOWLEDGEMENTS

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## EXPLANATION OF PLATES

### PLATE 1

FIG. 1. *Ericulus* (E 2—ser. 51*a*, 16-2-1), 33 somites. Section through developing mesonephros. Mesonephric tubules to the left, aorta to the right. Between the two (arrow) cellular proliferation from the coelomic epithelium extending into the subjacent mesoderm. ×300.

FIG. 2. *Hemicentetes* (H 1—ser. 38, 18-3-3), 26 somites. At arrow, a branching cord of cells projects from the coelomic epithelium into the mesoderm, between the aorta on the right and the mesonephros on the left. ×300.

FIG. 3. *Hemicentetes* (H 3—ser. 14*a*, 3-6-6), 4·75 mm. To the left of the aorta the vascularized cortical primordium can be seen. It is quite continuous below with the rostral part of the developing gonad. ×300.

FIG. 4. *Hemicentetes* (H 4—ser. 39, 20-2-4), 40 somites. In the centre, the aorta, flanked by the sympathetic. Ventrolaterally, on the left, a group of migrating paraganglion cells. On the right, the adrenal cortex in close relation with the gonad. ×75.

FIG. 5. *Hemicentetes* (H 4—ser. 39, 20-2-4), 40 somites. Higher magnification of left cortical primordium in fig. 4 to show intimate relation with the gonad (lower right corner). Protosympathetic in upper left corner. ×300.

FIG. 6. *Centetes* (Cl—ser. 50*a*, 17-2-3), *c*. 6 mm. To the right and left of the aorta, and ventrally, the cortical primordia with early invasion by paraganglion cells. On the left, the cortical primordium is apparently separated from the gonad, but the cleft is artificial; the two are still in contact. ×75.

### PLATE 2

FIG. 7. *Hemicentetes* (H 5—ser. 18, 11-4-2), 8·5 mm. Below (i.e. ventral to) the aorta, in the middle, is the large well-vascularized median paraganglion. Dorsolateral to the aorta, the sympathetic chain. ×80.

FIG. 8. *Hemicentetes* (H 6—ser. 11, 39-1-2), 8·5 mm. In the centre, the aorta. To the left, the cortical primordium in conjunction with paraganglionic tissue. On the right, the cortical primordium dorsal to the liver, shows nests of invading paraganglion cells. ×80.

FIG. 9. *Hemicentetes* (H 5—ser. 18, 15-3-3), 8·5 mm. The cortical primordium showing mitoses and cellular continuity with the coelomic epithelium. ×320.

FIG. 10. *Hemicentetes* (H 6—ser. 11, 45-2-3), 8·5 mm. The aorta is at the right margin of the figure. To the left of it is the sympathetic and migrating sympathoblasts. These lead to the cortical primordium, veins from which are opening into the adjacent cardinal vein. Cortical cells can be traced right to the hilum of the gonad (below). ×80.

FIG. 11. *Hemicentetes* (H 9—ser. 12, 14-3-3), 13 mm. Above (i.e. dorsally) is the aorta, with the large median paraganglionic mass in front of it. ×36.

FIG. 12. *Ericulus* (E 3—ser. 57, 54-2-5), 15 mm. Superolateral pole of paraganglionic mass (lighter staining) with thin investment of small dark cortical cells. At the upper margin of figure, part of the para-aortic paraganglionic column can be seen on the left. ×80.

### PLATE 3

FIG. 13. *Ericulus* (E 5—ser. 57, 17-1-5), 15 mm. The region of the upper pole of the superior cervical ganglion (dark cells on right), near base of skull (below). In upper left corner, paraganglionic mass—the upper part of the cervical column. ×50.

FIG. 14. *Ericulus* (E 5—ser. 57, 54-2-5), 15 mm. Dorsal para-aortic paraganglion becoming continuous with adrenal medulla, capped on left by cortical cells. In upper left-hand corner, the metanephros. ×50.

FIG. 15. *Ericulus* (E 7*b*—ser. 58*z*, 43-1-1), 26 mm. Dorsal thoracic wall, showing paraganglion column above (between arrows), and sympathetic below. ×22.

FIG. 16. *Ericulus* (E 4—ser. 74, 61-1-1), approx. 12 mm. Paraganglion column in cervical region

(upper left corner) with part of superior cervical ganglion (dark cells) adjacent. Also showing: below and to the left, thymus; to right, below, the thyroid, and above that, parathyroid IV. × 100.

Fig. 17. *Ericulus* (E 5—ser. 57, 23-2-2), 15 mm. Paraganglionic column (large cells) in conjunction with upper thoracic sympathetic ganglion (small dark cells). × 50.

Fig. 18. *Centetes* (C 4—ser. 20, 179-2), 21 mm. Cervical paraganglion (on right) at level of carotid fork. Above the internal carotid (cut longitudinally) is the carotid body, of quite different structure from the paraganglion. × 50.

Fig. 19. *Ericulus* (E 7*a*—ser. 58, 21-1-4), 26 mm. Rostral end of cervical paraganglion at carotid fork. From below: the cervical paraganglion (small), large superior cervical ganglion, carotid body (above and to right of sectioned internal carotid). × 50.

Fig. 20. *Ericulus* (E 7*b*—ser. 58*z*, 79-2-1), 26 mm. Paramedian sagittal section, showing the cap of dark cortical cells (above) over the paraganglionic mass. × 50.

Fig. 21. *Ericulus* (E 7*b*—ser. 58*z*, 79-2-1), 26 mm. Higher magnification of cortico-medullary junction in fig. 20. Cortex to right and below; medulla to left and above. × 200.

Fig. 22. *Centetes* (C 8—ser. 66*a*, 18), 69 mm. Section shows cortical differentiaton, with smaller cells at surface ('glomerulosa') and larger deeper cells ('fasciculo-reticularis'). Paler staining medullary cells are at the bottom of the figure. × 200.

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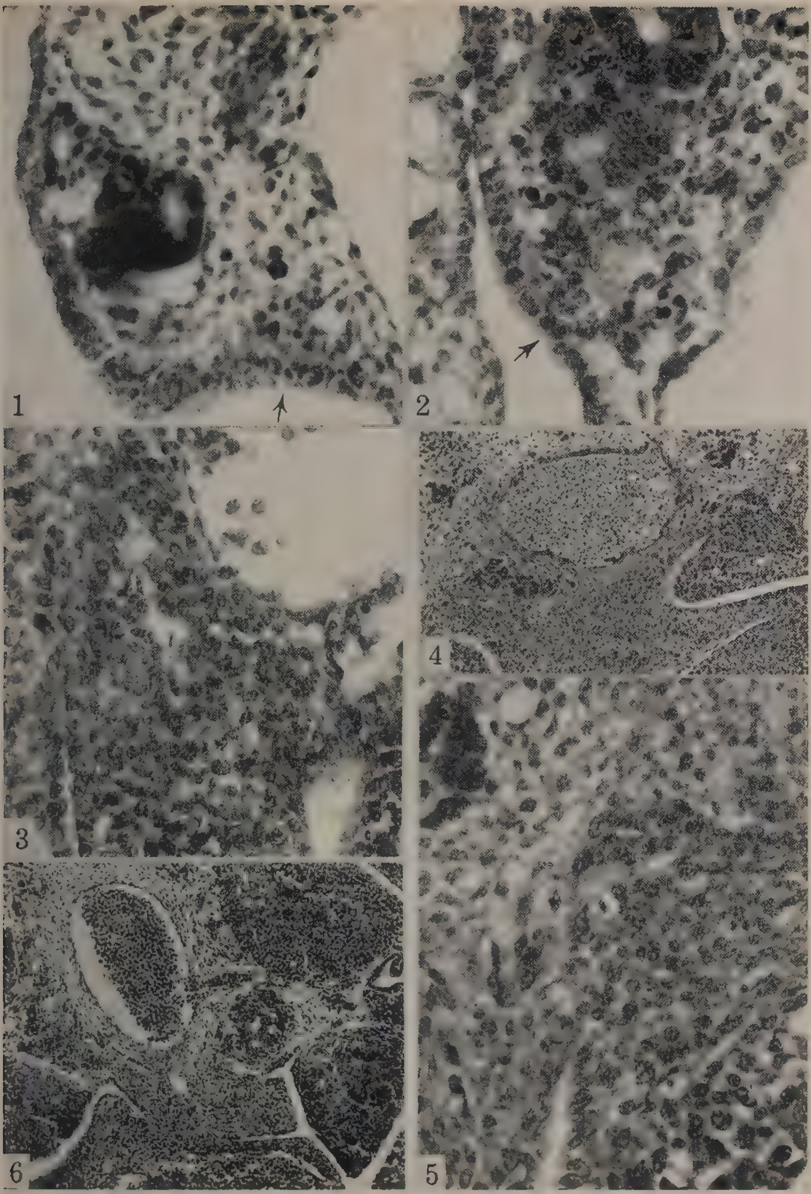

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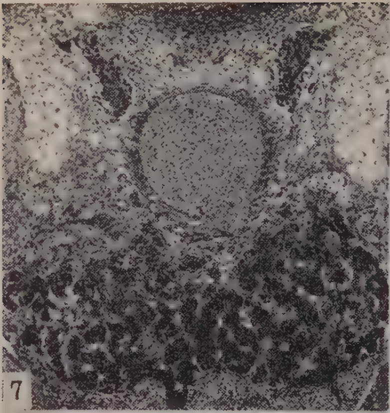
*Plate 1*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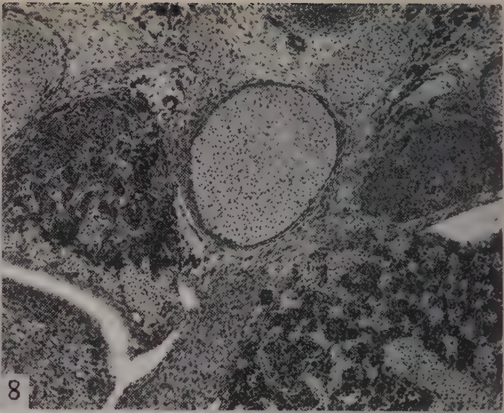
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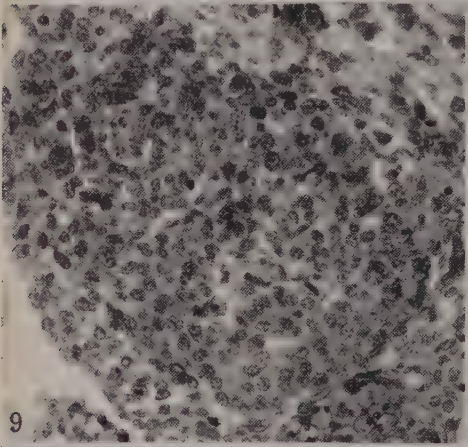
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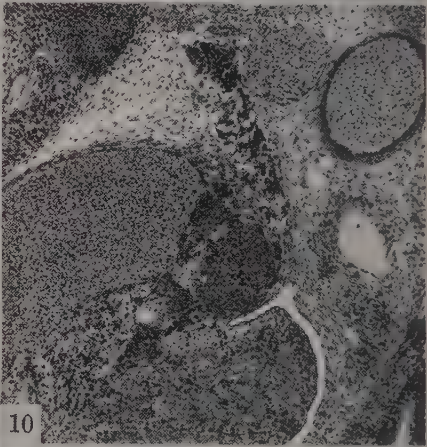
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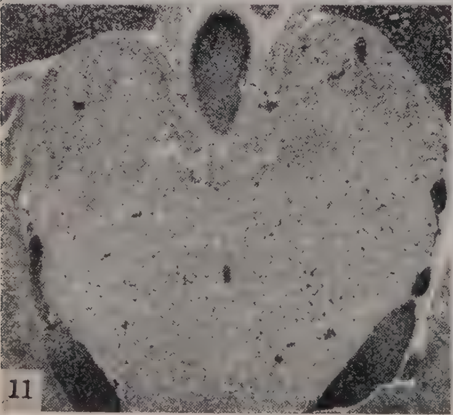
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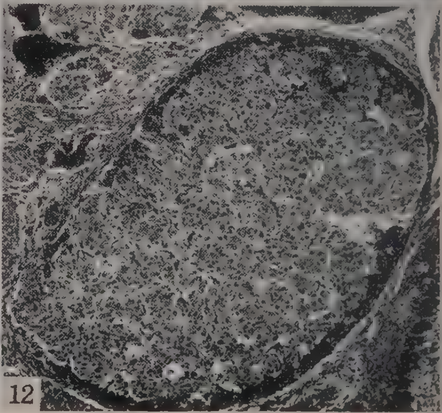
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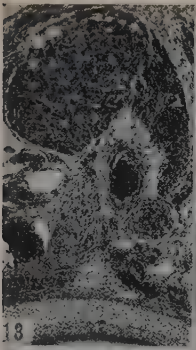
*Plate 2*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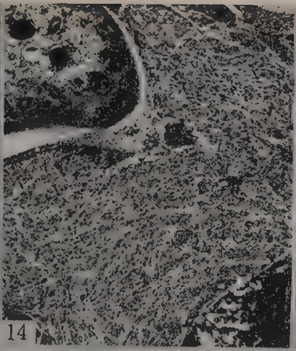
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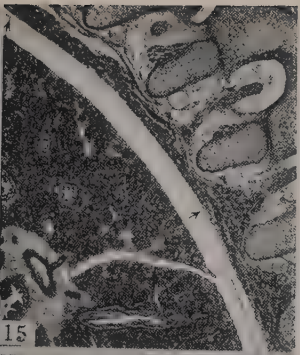
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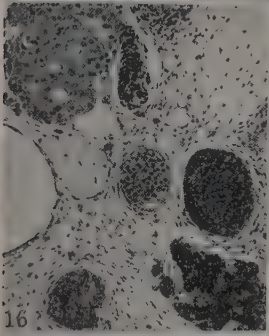
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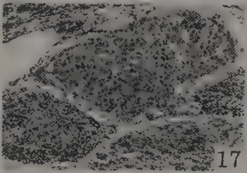
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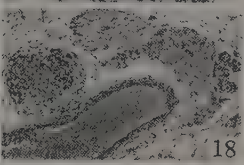
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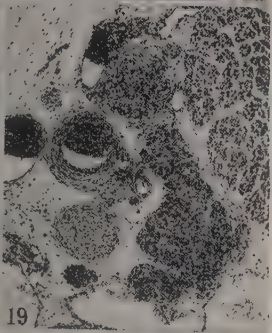
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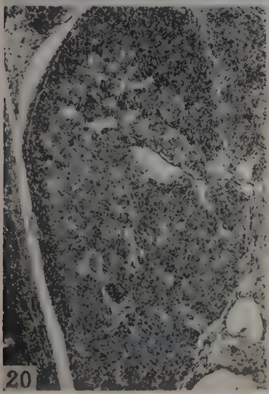
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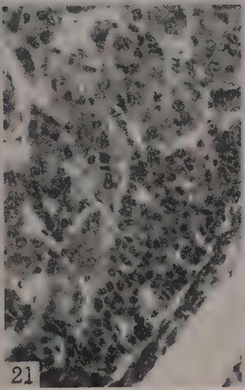
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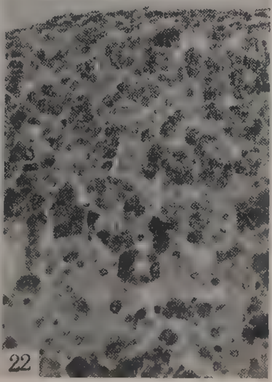
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*Plate 3*

# Inherited Retinal Dystrophy in the Mouse: its Appearance in Eyes and Retinae Cultured *in vitro*

*by* D. R. LUCAS[1]

*From the Wernher Group for Research in Ophthalmological Genetics*
*(Medical Research Council), Royal College of Surgeons*

WITH TWO PLATES

CERTAIN strains of mice, such as C3H, exhibit a recessively inherited retinal dystrophy. This takes the form of a degeneration and complete disappearance of the visual cell layer; it begins on or about the 11th day of life and is nearly complete by the 19th day (Sorsby, Koller, Attfield, Davey, & Lucas, 1954). The process is remarkably uniform from litter to litter (Lucas & Newhouse, 1957).

In a previous paper (Lucas & Trowell, 1958) it was shown that eyes and retinae taken from 10-day-old normal (CBA) mice could be maintained *in vitro* for 8 days, during which time their structure was fairly well preserved and differentiation proceeded.

In the present work, eyes and retinae from dystrophic strains of mice have been cultured over a similar period to see if the dystrophy developed *in vitro*.

## MATERIAL

The eyes or retinae of 25 animals from 3 litters of the C3H strain, maintained at the M.R.C. Radiobiological Unit, Harwell, and from 2 litters of the retinal dystrophy (Brückner) strain maintained at the Royal College of Surgeons, were explanted. The retinal condition is histologically identical in the two strains and in $F_1$ hybrids. Details of breeding experiments showing the genetic identity of the two strains have been reported elsewhere (Lucas, 1958).

Two groups of mice were used, the one 9–10 days old (12 animals), the other 11–12 days old (13 animals). Retinae from a number of normal CBA-mice of the stock maintained at Harwell were explanted as *in vitro* controls.

## METHODS

The cultural and histological methods were as described in the previous paper (Lucas & Trowell, 1958). Each experiment was performed on a group of littermates. As controls, the eyes of one mouse were fixed when the cultures were set

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up ('initial' controls), and one animal was set aside to be killed at the end of the experiment and provide *in vivo* controls. The eyes or retinae of the remaining available mice were cultured for 7 days. In most experiments, cultures were also made of normal eyes or retinae from CBA-mice as near the same age as possible.

## RESULTS

In general, the cultures were equal in quality to those already reported (Lucas & Trowell, 1958); the isolated retinae showed some loss of cells from all layers and in the whole eyes there was usually some necrosis of the retina at the posterior pole. Retinal malformations also developed in the cultured eyes with consequent variation in the relative thickness of the different layers. These factors made the results more difficult to interpret, but a fairly clear picture nevertheless emerged.

*Mice aged 11–12 days.* The initial controls showed slight thinning of the outer nuclear layer in the area centralis and some of the nuclei were pyknotic indicating that the dystrophy had already begun (Plate 1, fig. A). At the periphery the outer nuclear layer was of normal thickness (Plate 1, fig. B). In all the cultured eyes except two, which were too necrotic to interpret, the outer nuclear layer was distinctly thinner than in the initial controls, but not so thin as in the *in vivo* controls (Plate 1, figs. C, D). Also, in the cultured C3H eyes the outer nuclear layer was thinner than in cultured CBA eyes (Plate 2, fig. E).

The retinal cultures (from 2 eyes) in this group showed almost complete disappearance of the visual cell layer (Plate 2, fig. F).

*Mice aged 9–10 days.* In the initial controls the outer nuclear layer appeared normal except that it contained very occasional pyknotic nuclei. In 5 of the 15 cultured eyes the outer nuclear layer showed definite thinning as compared with parallel cultures from normal CBA-mice. It was, however, much thicker than in the *in vivo* controls. In the other 10 eyes in this group it was impossible to be sure that any thinning had occurred because of the malformation or necrosis of the retina referred to above.

In the 4 isolated retinae cultured in this group, the number of visual cells surviving was not consistently lower than in parallel cultures of CBA-mice.

It appeared, therefore, that the dystrophy proceeded during culture, though at a slower rate than *in vivo*. It could be demonstrated more consistently when it had already begun before culture as in the 11–12-day group.

## DISCUSSION

The results show that, under the condition of these experiments, the degeneration and disappearance of the visual cell layer which normally take place between 11 and 19 days in mice of a retinal dystrophy strain are considerably delayed. This delay is probably due to retarded differentiation. It has already been found that the onset of degeneration in the intact animal is delayed under conditions of malnutrition severe enough to interfere with retinal differentiation (Lucas & Newhouse, 1957). Differentiation of the visual cells under present conditions is undoubtedly abnormal since, though the nuclei show the changes normally observed *in vivo*, the rods fail to elongate (Lucas & Trowell, 1958). It is also possible, however, that there is a humoral factor in the affected animal from the influence of which the cultures have been removed. Wolff & Kieny (1955) have recently demonstrated the existence of a humoral factor in extracts of Creeper fowl embryos which retards the growth of tibial explants from both normal and heterozygous Creeper embryos when added to the culture medium.

## SUMMARY

Using an organ culture technique the eyes and retinae of mice affected by an inherited retinal dystrophy were maintained for 7 days *in vitro*. The rate of the dystrophic process was considerably retarded as compared with the living animal.

## ACKNOWLEDGEMENT

I should like to thank Dr. J. F. Loutit for making available facilities in the M.R.C. Radiobiological Unit, Harwell, and Dr. O. A. Trowell for much advice and encouragement.

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## EXPLANATION OF PLATES

### PLATE 1

FIG. A. Initial control. Area centralis of C3H mouse aged 12 days showing slight thinning of outer nuclear layer and occasional pyknotic nuclei; fixed acid Zenker. ×400.

FIG. B. Initial control. Periphery of retina of same eye as in fig. A showing normal outer nuclear layer (the raggedness is an artefact). ×200.

FIG. C. Periphery of retina of eye of C3H mouse aged 12 days (litter-mate of mouse from which eye in figs. A and B originated) after 7 days in culture showing thinning of outer nuclear layer; fixed acid Zenker. ×200.

FIG. D. *In vivo* control. Periphery of retina of eye of C3H mouse aged 19 days (litter-mate of mice from which eyes in figs. A, B, and C originated) showing remnants only of the visual cell layer; fixed acid Zenker. ×200.

PLATE 2

FIG. E. *In vitro* control. Periphery of retina of eye of CBA-mouse aged 10 days and cultured in same chamber as eye shown in fig. C showing normal appearance of outer nuclear layer after 7 days in culture; fixed acid Zenker. × 200.

FIG. F. Retina of C3H mouse aged 12 days after 7 days in culture, showing loss of nearly all the visual cell layer. Cf. fig. A of which the mouse was a litter-mate; fixed formal sublimate. × 400.

FIG. G. *In vitro* control. Retina of CBA-mouse aged 10 days after 7 days in culture showing amount of visual cell layer normally preserved; fixed formal sublimate. × 400.

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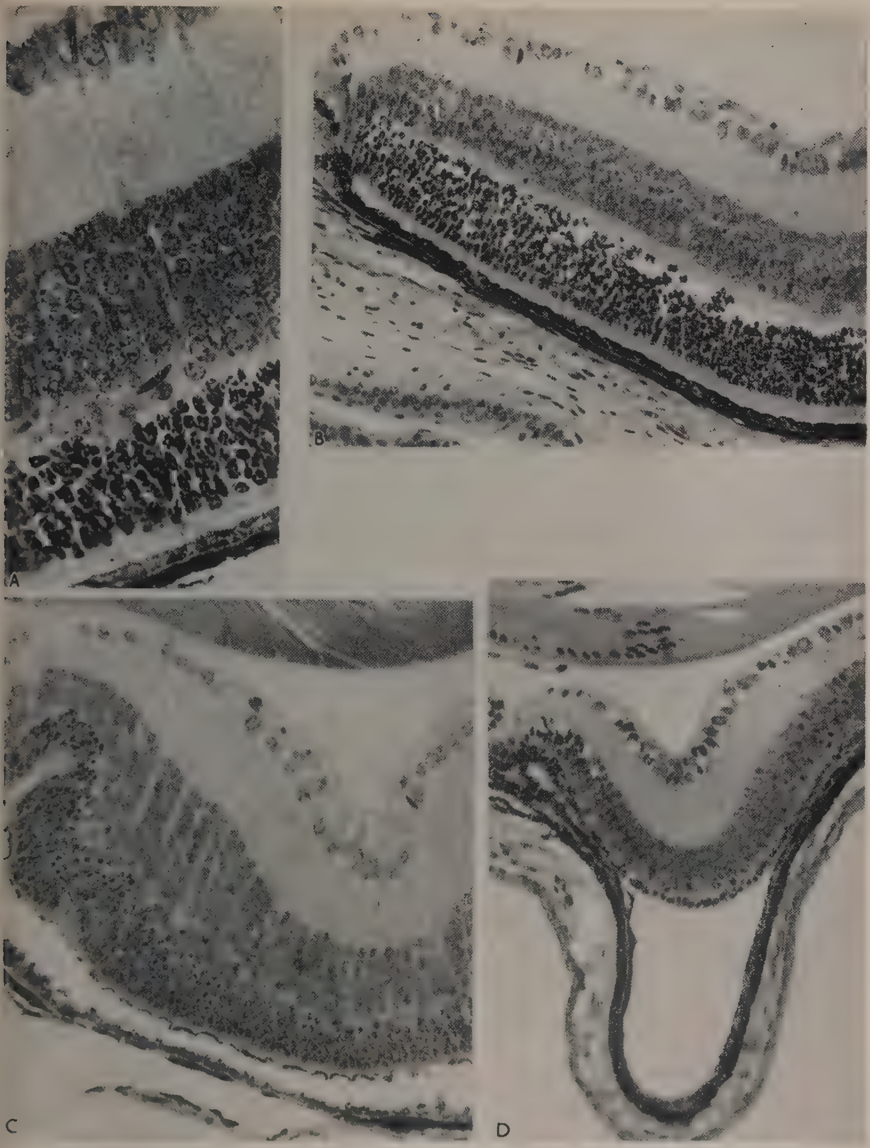
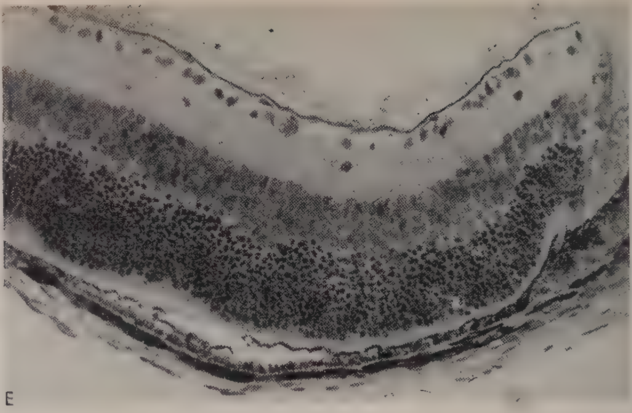
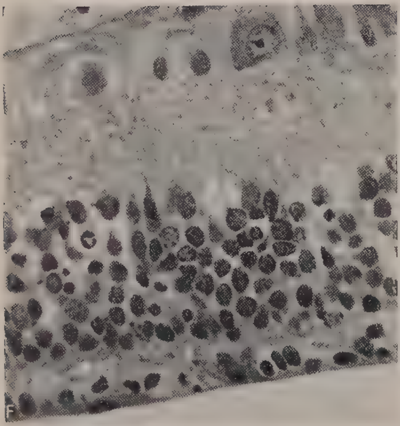
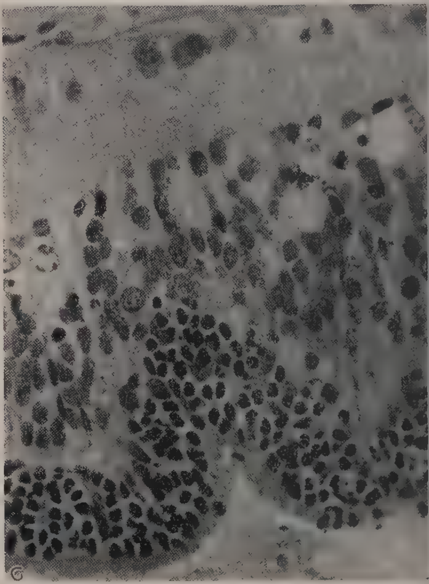


D. R. LUCAS

*Plate 1*







D. R. LUCAS

*Plate 2*

# Congenital Malformations in Mouse Embryos Induced by 8-Azaguanine

*by* HIDEO NISHIMURA *and* HIROAKI NIMURA[1]
*From the Department of Anatomy, Kyoto University*

## INTRODUCTION

IN recent years congenital malformations have been induced in higher mammals by such exogenous factors as nutritional deficiencies, anoxia, certain hormones, and chemicals (Fraser & Fainstat, 1951; Hickey, 1952; Wilson, 1954; Nishimura, 1956). This study was undertaken to ascertain whether 8-azaguanine, an antagonist to guanine, has a teratogenic effect on mouse embryos.

## METHOD

Female mice of the Japanese *dd* strain were mated at 3 to 5 months of age. On days 7 to 15 of pregnancy they were given a single intraperitoneal injection of 8-azaguanine solution (16 mg./c.c.) and then were sacrificed shortly before term to determine state of pregnancy and condition of the foetuses. Routine histological examinations were made of malformed organs and also of the placentae of affected embryos. As controls foetuses from 30 untreated mothers from the same colony were examined.

## RESULT

Table 1 shows the results of injection with 8-azaguanine at various times in gestation.

Of 245 control foetuses from untreated mice, only 8 were dead and one malformed (with double ungual process of the first digit of the hind foot). It is clear, therefore, that 8-azaguanine often has a lethal effect. Many embryos seem to have died and become macerated immediately after injection as indicated by the frequency of complete resorptions found at term. Where pregnancy was not interrupted by complete resorption no remarkable alteration in litter size was found.

It is also clear that 8-azaguanine has a considerable teratogenic effect. The malformations occurred mostly in the skeletal system, and especially in the extremities, although there were some cases of cleft palate. Histologically it

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was observed that the deviated digits, the commonest type of anomaly, were associated with malformed joints and sometimes also with bent proximal bones. In cases of *pes varus* or *pes valgus*, luxation of the talocrural joint, hydrops of the tarsal joint with atrophy of the tarsal bone cells or irregularity in form of metatarsal bones was observed. All cleft palates were bilateral; some were incomplete posteriorly, others complete. It should be noted that the critical period for digital anomalies, if one exists, covered a lengthy period, from the 8th to at least the 15th day. No apparent alteration of body-weight was recognized in the living embryos subjected to 8-azaguanine. Placentae of malformed foetuses showed no macroscopic or histological abnormalities.

TABLE 1

*Effects of a single injection of 8-azaguanine on pregnant mice*

| *Days of injection* | *Dosage* (mg./g.) | *Total No. of mice* (*litters*) | *No. of pregnancies undergoing complete resorption* | *Foetuses at term* | | | *Types and No. of anomalies** |
|---|---|---|---|---|---|---|---|
| | | | | *Total No.* | *No. dead* | *No. with malformation* | |
| 7 | 0·08 | 4 | 4 | — | — | — | — |
| 7 | 0·24 | 5 | 3 | 10 | 0 | 0 | — |
| 8 | 0·08 | 2 | 0 | 12 | 0 | 4 | 1 Ps, 1 Cd, 2 Pd |
| 8 | 0·24 | 7 | 4 | 22 | 0 | 5 | 1 Ps, 4 Pd, 1 Sd(t), 1 Dd(t) |
| 8 | 0·32 | 2 | 2 | — | — | — | — |
| 8 | 0·4 | 1 | 0 | 6 | 0 | 1 | 1 Md(t) |
| 9 | 0·24 | 1 | 1 | — | — | — | — |
| 9 | 0·32 | 2 | 2 | — | — | — | — |
| 9 | 0·4 | 1 | 0 | 8 | 0 | 5 | 1 Bd(f), 1 Sd(f), 2 Dd(f), 5 Dd(t) |
| 10 | 0·32 | 2 | 1 | 2 | 2 | — | — |
| 11 | 0·24 | 5 | 2 | 20 | 0 | 2 | 2 Sd(t) |
| 11 | 0·32 | 1 | 0 | 7 | 0 | 1 | 1 Sd(t) |
| 11 | 0·4 | 4 | 3 | 7 | 7 | — | — |
| 12 | 0·24 | 2 | 1 | 8 | 1 | 3 | 3 Dd(t), 1 Dd(f) |
| 12 | 0·32 | 4 | 4 | — | — | — | — |
| 12 | 0·4 | 5 | 4 | 7 | 1 | 4 | 2 Ps, 4 Dd(t) |
| 13 | 0·32 | 2 | 0 | 13 | 0 | 4 | 6 Dd(t) |
| 13 | 0·4 | 1 | 1 | — | — | — | — |
| 14 | 0·32 | 1 | 0 | 7 | 0 | 2 | 2 Dd(t) |
| 14 | 0·4 | 1 | 0 | 8 | 0 | 2 | 3 Dd(t) |
| 15 | 0·32 | 3 | 0 | 19 | 0 | 4 | 4 Dd(t) |

* Bd(f): brachydactyly (finger). Bd(t): brachydactyly (toe). Cd: malformation of elbow joint. Dd(f): deviation of finger. Dd(t): deviation of toe. Md(t): macrodactyly (toe). Pd: malformation of ankle joint. Ps: cleft palate. Sd(f): syndactyly (finger). Sd(t): syndactyly (toe).

## DISCUSSION

Concerning the mechanism of the teratogenic effect of 8-azaguanine little can be said, except that there was no evidence that the placenta was implicated.

The fact that it induced malformations mostly in the skeletal system recalls the results obtained with such agents as nitrogen mustard (Haskin, 1948; Danforth & Center, 1954; Thalhammer & Heller-Szöllösy, 1955; Takagaki, 1957), and ethylurethan (Sinclair, 1950; Nishimura & Kuginuki, 1958). However, 8-azaguanine is peculiar in that it caused numerous slight malformations such as deviation of interphalangeal joints.

## SUMMARY

1. The effect of 8-azaguanine on the development of offspring of mice was investigated by injecting mothers once intraperitoneally with 0·08 to 0·4 mg./g. of body-weight on a day between the 7th and 15th of gestation.

2. The principal teratogenic effect was on the skeletal system. Among the malformations found were cleft palates and various abnormalities of the extremities; abnormally directed digits due to deviation of interphalangeal joints were most frequently encountered.

3. When administered between the 7th and 14th days of gestation, this compound is frequently lethal to embryos, causing death immediately or a few days after injection.

## ACKNOWLEDGEMENTS

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# The Origin of the Acoustic Ganglion in the Sheep

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*From the Department of Physiology, University of Bristol*

WITH TWO PLATES

## INTRODUCTION

It has long been customary to regard the VII and VIII ganglia in the mammal as originating from a common primordium—the acustico-facial crest—probably owing to their almost simultaneous appearance and close apposition during development (Adelmann, 1925; Bartelmez, 1922; Holmdahl, 1934; Kolmer, 1928; Politzer, 1928; Völker, 1922; and Weigner, 1901). But as long ago as 1906 Streeter drew attention to the early independence of the VII and VIII ganglia in the human embryo and suggested that their close intimacy should be regarded as contiguity rather than fusion. While he did not investigate the source of the acoustic ganglion cells Streeter (1906) questioned the accepted view of their origin from a common primordium and later (Streeter, 1912) stated that they were not derived from the neural crest. The resolution of this problem clearly involves the examination of a close series of embryos, particularly at stages earlier than that at which these ganglia appear to be fused. Thus in amphibia, where material is abundant, the formation of the acoustic ganglion from placodal cells which detach from the walls of the otic vesicle has been traced in studies of normal development (Brachet, 1907; Knouff, 1927, 1935; Kostir, 1924; and Niessing, 1932). Moreover, the results of experimental work strongly support this view since the formation of a normal acoustic ganglion is dependent on an intact otic vesicle and is not disturbed by extirpation of the adjacent facial and glossopharyngeal neural crest (Campenhout, 1935*a*; Stone, 1922; and Tokura, 1925). Nevertheless, Campenhout has admitted that this work does not absolutely prove that the ganglion arises from the otic vesicle as the neural crest is known to possess a marked regenerative ability. Yntema (1937) employed a Nile blue sulphate method for tracing the migrating placodal cells and showed that the acoustic ganglion is derived from the otic epithelium.

Few critical investigations into the origin of the acoustic ganglion have been carried out in mammals, primarily on account of the difficulty of obtaining sufficient embryos at an early stage of development. Campenhout (1935*b*) in the pig and Groth (1939) in the rabbit both describe an exclusively placodal origin for

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this ganglion. More recently, Halley (1955) found that the acoustic ganglion in the cat develops in large part from placodal cells and Politzer (1956) has traced the independent anlage of the same ganglion in the human to cells which proliferate from the wall of the otic vesicle. A recent study in the sheep (Batten, 1957) indicates that the VII and VIII ganglia have an independent origin, for the facial crest is entirely devoted to the formation of the geniculate ganglion, and this receives contributions from an epibranchial placode for some time before the enlarging acoustic ganglion fuses with it to produce the so-called acustico-facial complex. The present paper describes the formation of the acoustic ganglion from cells which detach from the placodal face of the vesicle and offers an explanation for the marked inequality in cytological differentiation which distinguishes the young acoustic neuroblasts from those of the adjacent geniculate ganglion.

## MATERIALS AND METHODS

Serial sections of 108 sheep embryos have been examined (Table 1). Thirty of these embryos belong to the collection of Professor J. D. Boyd and were cut at 6 μ and stained either in Weigert's haematoxylin and orange G, or in Heidenhain's haematoxylin alone. A further twenty specimens were given by Professor E. C. Amoroso and the remainder were collected locally. Bouin's fluid has been routinely used and, after paraffin embedding, the embryos were cut at 7 μ, or at 10 μ for reconstruction, and stained in Ehrlich's haematoxylin and eosin. The later embryos of 26 mm., 28 mm., and 35 mm. were prepared by Miss P. Morgan and loaned by Dr. R. N. Smith.

TABLE 1

*Description of material used*

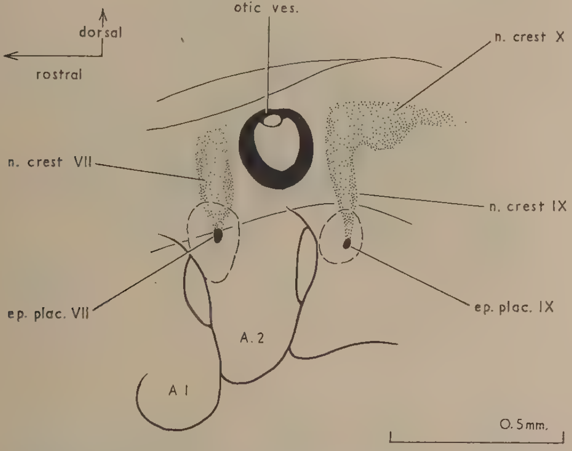
| *Stage (crown-rump length in mm.)* | *Age (in days)* | *Number of embryos examined* |
|---|---|---|
| 11–38 somites | 15–21 | 30 |
| 8·0–9·2 | 22–23 | 26 |
| 9·5–10·5 | 23–24 | 18 |
| 11·0–12·7 | 25–27 | 21 |
| 13·0–16·0 | 27–28 | 9 |
| 22·0–35·0 | 30–35 | 4 |
| TOTAL . . . . | | 108 |

The text-figures are drawings taken from graphic reconstructions of the otic vesicle and acustico-facial complex in selected embryos. Tracings were made at a magnification of 50 or 100 diameters using a section interval of 20 μ and the detail was transferred to a drawing board. By this method the size and distribution of the placodal spurs may be reconstructed with tolerable accuracy.

## RESULTS

### *12-somite—8·0 mm. embryos (15–22 days)*

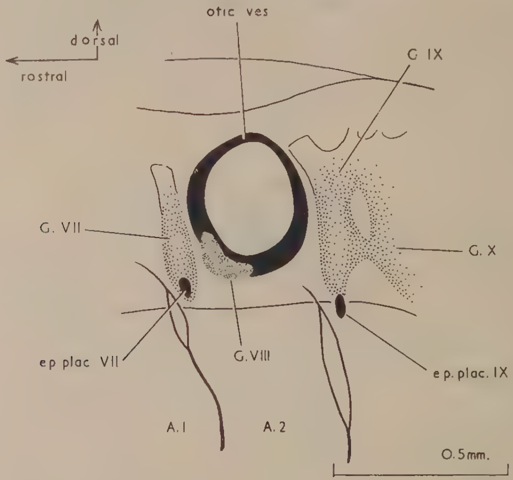
The facial neural crest can be identified as a diffuse cellular column as early as the 12–13-somite stage, when the neural tube is still open at this level and the otic placode is merely a flat plate of slightly thicker ectoderm. The formation of a thick-walled otic vesicle is completed by the 26-somite (19-day) stage and the transient connexion of the neck region with a disk of surface ectoderm is lost slightly later (Text-fig. 1). Concurrently, the epibranchial placode of the facial nerve enters a phase of active proliferation and the developing geniculate ganglion continues to receive intermittently small cellular contributions until the 10·5 mm. stage (Batten, 1957). The differentiation of the geniculate ganglion thus begins before there is any indication of the formation of an acoustic ganglion. Moreover, the effect of this developmental precedence of the facial nerve becomes evident later in the marked superiority in cytological differentiation which distinguishes the facial from the acoustic part of the VII–VIII complex.



TEXT-FIG. 1. Graphic reconstruction of the otic vesicle and adjacent ganglia in a 26-somite embryo. A. 1, mandibular arch; A. 2, hyoid arch; ep. plac. VII, facial epibranchial placode; ep. plac. IX, glossopharyngeal epibranchial placode; n. crest VII, facial neural crest; n. crest IX, glossopharyngeal neural crest; n. crest X, vagal neural crest; otic ves., otic vesicle.

By the 6·0-mm. (32-somite) stage the otic vesicle has grown considerably and now has thinner walls with numerous mitotic figures in their inner layer (Text-fig. 2). The basement membrane is clearly defined except along the ventral border where two small streams of cells lying close against the rostral and lateral external surface converge to unite in a common ventral mass. This mass represents the primordium of the acoustic ganglion and is separated from the developing geniculate ganglion by a zone of mesenchyme.

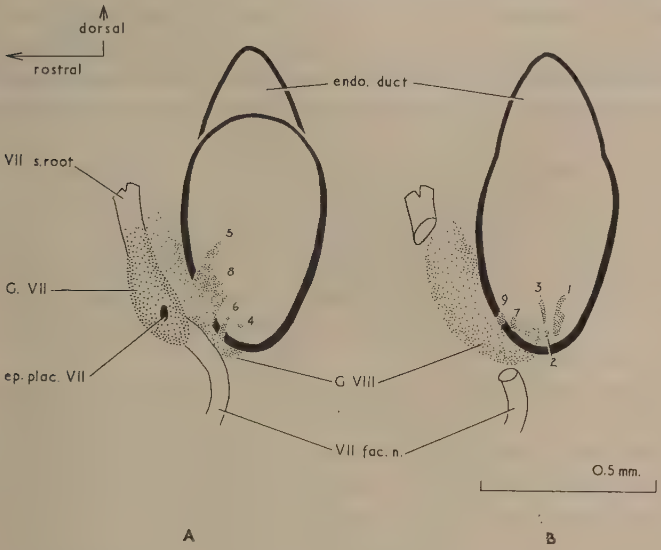
The early streams of placodal cells may be distinguished from mesenchyme by their intimate contact with the otic epithelium and more closely packed nuclei, and from the otic epithelium itself by nuclear orientation: the epithelial nuclei are arranged mainly in a radial pattern whereas the placodal nuclei usually lie at right angles to this and appear to be sweeping ventrally towards the acoustic ganglion (Plate 1, figs. A, B). Although similar flat streams of cells leading to a minute acoustic ganglion were observed in six other embryos with 30–33 somites there is no clear indication of their precise origin. The first convincing evidence of the emergence of these placodal streams from the otic epithelium was found in a 7·2-mm. (36-somite) embryo with six distinct streams on the right side. Two streams projected from the lateral wall of the vesicle as short free-ending papillae, and of the four medial streams one was free-ending and the others traced ventrally to unite with the acoustic ganglion. An early stage in the migration of placodal cells from the otic epithelium is shown in Plate 1, fig. A 1; this slender stream of cells appears to be interposed between the epithelium and its basement membrane, but ends without penetrating the membrane after a distance of 30 $\mu$ in serial section. The larger spur (Plate 1, fig. A 2) can be



TEXT-FIG. 2. Graphic reconstruction of the otic vesicle and adjacent ganglia in a 6·0-mm. (32-somite) embryo. A. 1, mandibular arch; A. 2, hyoid arch; ep. plac. VII, facial epibranchial placode; ep. plac. IX, glossopharyngeal epibranchial placode; G. VII, geniculate ganglion; G. VIII, placodal rudiment of acoustic ganglion; G. IX, glossopharyngeal ganglion; G. X, vagal ganglion; otic ves., otic vesicle.

followed for 50 $\mu$ before it enters the edge of the acoustic ganglion. Unlike the smaller spur, it is not covered by basement membrane, but lies free in a shallow furrow in the basal layer of the otic epithelium. Two other spurs have also broken through the membrane and extend ventrally to connect with the acoustic ganglion.

By the 8·0-mm. stage the otic vesicle has grown into a pyriform sac which bears along its thin dorso-medial border a flattened rudiment of the endolymphatic duct. The acoustic ganglion itself is also much larger and now partially overlaps the geniculate ganglion and spreads along its root to reach the hind-brain (Text-fig. 3A). Since, however, the facial and acoustic ganglia exist as separate primordia in the previous stage their secondary union to produce the acustico-facial complex has no developmental significance and should be regarded as the sequel to a phase of rapid differential growth of the acoustic ganglion.



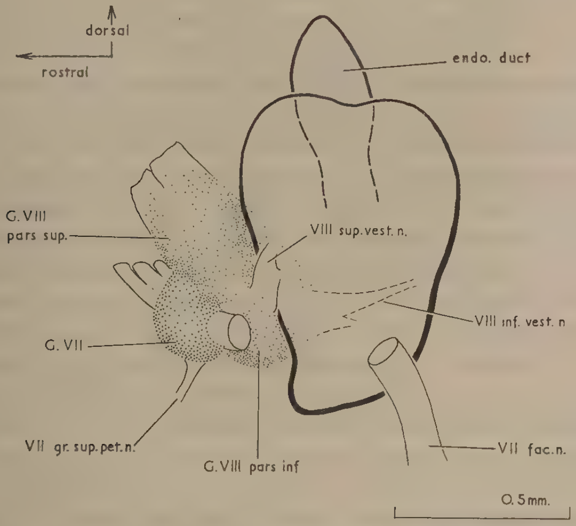
TEXT-FIG. 3. Graphic reconstruction of the otic vesicle and acustico-facial complex in an 8·0-mm. embryo. A: showing the placodal spurs (numbered) present on the lateral wall of the vesicle. B: showing the placodal spurs present on the medial wall, the lateral half of the vesicle and the geniculate ganglion having been omitted to show the extent of the acoustic ganglion. endo. duct, endolymphatic duct; ep. plac. VII, facial epibranchial placode; G. VII, geniculate ganglion; G. VIII, acoustic ganglion; VII fac. n., facial nerve; VII s. root, sensory root of facial nerve.

The main bulk of the acoustic ganglion lies close against the rostral face of the ventral part of the vesicle and sends a short extension behind its medial wall (Text-fig. 3B and Plate 2, fig. G). The rapid growth of the acoustic ganglion is due to the increment of placodal cells which detach in small streams from the lateral and medial walls of the vesicle. Text-fig. 3 illustrates the size and distribution of nine placodal spurs present in an 8·0-mm. embryo. The migration of cells from the lateral wall is particularly active and the two larger streams of placodal cells (spurs 5 and 8 in Text-fig. 3A) may be traced ventrally to connect with the acoustic ganglion. Both the smaller spurs are free-ending: spur 6 is a slender file of cells and spur 4 a short papilla which probably represents a site of incipient detachment. The medial wall of the vesicle bears five placodal spurs all of the free-ending type representing sites of active cellular detachment. The cells of the largest example (spur 1 of Text-fig. 3B) appear to be streaming from a breach in the basement membrane, but have not yet reached the acoustic ganglion. Two smaller spurs (2 and 3 of Text-fig. 3B) are shown in section in Plate 1, fig. B. In shape the upper spur (Plate 1, fig. B 3) is a slender file which projects ventrally towards the acoustic ganglion, the extreme caudal tip of which is also intercepted in this section. The cells of this file are still enclosed in a tubular extension of basement membrane, but this appears to be broken distally at the free end. The lower spur (Plate 1, fig. B 4) is probably in the act of detaching from the otic epithelium since it lacks a covering of basement membrane which is well preserved on either side.

Examination of other embryos of similar age indicates that the detachment of cells from the lateral wall is more vigorous than from the medial wall. The otic epithelium no longer possesses a smooth contour, but is strewn with small irregular clusters of cells; some of these are entirely free and in the course of their ventral migration (Plate 1, fig. C 5 and 6); others are about to detach from the epithelium (Plate 1, fig. C 7 and 8). Of 14 sites of cellular detachment in this 8·2-mm. embryo, 9 were related to the lateral and 5 to the medial wall of the vesicle. Even at low magnification the groups of migrating placodal cells are conspicuous. In a typical coronal section through the middle of the vesicle the placodal spurs stand out as thin streams of deeply staining cells lying close against the lateral wall of the vesicle (Plate 1, fig. D). When traced rostrally for a distance of 40 μ these streams lead into the acoustic ganglion which lies at the ventral border of the vesicle and receives from it a further placodal increment (Plate 1, fig. E 9). A small site of impending cellular detachment is seen on the lateral wall of the vesicle in Plate 1, fig. E 10. After the enlarging acoustic ganglion unites with the geniculate ganglion just before the 8·0-mm. stage the two components still retain their individuality through cytological differences. The young neuroblasts of the geniculate ganglion contrast even under low magnification with the smaller and more basophilic nuclei of the tightly packed placodal cells of the acoustic ganglion (Plate 2, figs. F, G). While coronal sections indicate that cells detach from the lateral and medial walls of the vesicle, it is evident in transverse sections that the rostral wall, against which the acoustic ganglion closely abuts, is also placodally active. Three active spurs are shown in Plate 2, fig. F; the largest of these (spur 11) forms a connecting bridge between the otic epithelium and the ganglion, while the other two (spurs 12 and 13) are smaller sites of impending cellular detachment. In a section through the base of the vesicle in the same 8·0-mm. embryo, the acoustic ganglion extends a short distance along the medial wall and receives from it the two massive placodal streams (Plate 2, fig. G 14 and 15). In this embryo 10 spurs were counted, 3 along the medial wall and 7 against the rostral and lateral walls.

### *9·0–11·0-mm. embryos (22–25 days)*

Up to the 10·0-mm. stage placodal spurs continue to form on the walls of the vesicle and the detached cells emigrate into the enlarging acoustic ganglion. The number of spurs recorded in these embryos usually lies between eight and ten, the majority being small free-ending buds and the remainder larger streams of the connecting type. The buds which proliferate from the lateral and rostral sides of the vesicle contribute to the dorsal half of the ganglion which retains an intimate contact with the dorso-medial surface of the geniculate ganglion. As a result of active budding from the ventro-medial wall the ventral half of the acoustic ganglion gradually enlarges both in a ventral and in a medial direction to produce a marked extension behind the otic vesicle (cf. Plate 2, figs. G, I).



TEXT-FIG. 4. Graphic reconstruction of the otic vesicle, acoustic and facial nerves in an 11·0-mm. embryo. endo. duct, endolymphatic duct; G. VII, geniculate ganglion; G. VIII pars inf., pars inferior of acoustic ganglion; G. VIII pars sup., pars superior of acoustic ganglion; VII fac. n., facial nerve; VII gr. sup. pet. n., greater superficial petrosal nerve; VIII inf. vest. n., inferior vestibular nerve; VIII sup. vest. n., superior vestibular nerve.

About this stage the ganglion begins to show a partial subdivision which so closely resembles that described in the human by Streeter (1906) that his terminology may conveniently be applied. Thus the dorsal part of the acoustic ganglion, which lies close against the rostral wall of the vesicle and overrides the geniculate ganglion, may be termed the pars superior (Text-fig. 4 and Plate 2, fig. H).

In comparison with the 8·0-mm. stage this part of the ganglion shows a vigorous dorsal growth and is now connected with the brain by a broad fibrous root which overlaps and obscures from surface view the slender sensory root of the facial nerve (Text-fig. 4 and Plate 2, fig. H). The remaining ventral part of the ganglion together with its medial extension behind the base of the vesicle constitutes the pars inferior (Text-fig. 4 and Plate 2, fig. I).

During the 9·0-mm. stage the small deeply basophilic nuclei of the placodal cells give the pars inferior an even appearance, but by the 9·5–10·0-mm. stage there are signs of a cytological differentiation which subdivides this part into two regions. The nuclei of the lateral region which lies against the otic vesicle become larger and elongate in a rostro-caudal plane. As a study of later stages reveals, this change marks the beginning of the differentiation of these placodal cells into neuroblasts. By contrast the cells of the medial region of the pars inferior retain for some time the appearance of typical small placodal elements. In transverse sections the distinction between the fusiform region of young vestibular neuroblasts and the residual placodal cells of the medial region facing the brain wall is an impressive feature (Plate 2, fig. I). The contrast between the facial and acoustic ganglia at this ventral level is accentuated by the more advanced differentiation of the geniculate neuroblasts which are of crest origin. The bulk of the geniculate ganglion now overhangs the rostral face of the motor trunk and thus begins to assume its characteristic pendant form.

Shortly after the onset of this regional differentiation the caudal edge of the pars inferior bears a short fibrous extension which closely follows the basement membrane of the otic epithelium. Although specific silver methods have not been employed there is little doubt that this fibrous extension consists of nerve-fibres for its structure under oil immersion examination is identical with that of adjacent cranial nerves. When the development of this outgrowth is traced back from the later stages it can be identified as the earliest rudiment of the inferior vestibular nerve. Initially this fine branch ends against the otic epithelium at a point near the middle of the medial wall of the vesicle, as in Text-fig. 4 and Plate 2, fig. I, but in other 11·0-mm. embryos it can be followed as a fine strand of delicate fibres into the caudal edge of the vesicle at a level where the ampulla of the posterior canal will later develop. The rudiment of the superior vestibular nerve regularly appears slightly later as a short fibrous stump connecting the pars superior with the rostral margin of the otic vesicle (Text-fig. 4). Since it is rather finer than the inferior vestibular branch it is less easy to photograph satisfactorily at this stage, but its position is indicated in Plate 2, fig. H.

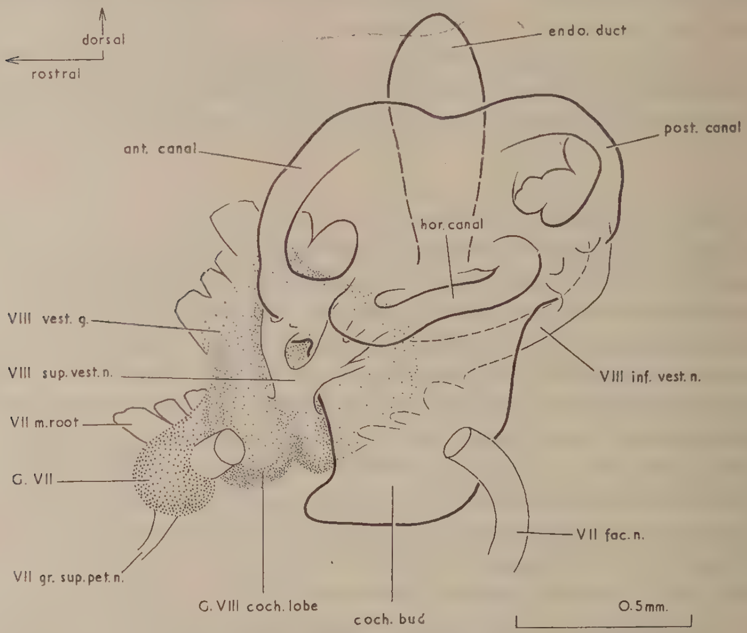
During the 10·5-mm. stage there is a decline in the extent of placodal activity of the rostral and medial walls of the vesicle, for most embryos show only two or three buds of small size. A typical example which is about to detach and migrate into the pars superior is seen in Plate 2, fig. H 16. By the 11·0-mm. stage there is little further evidence of contributions to the pars superior, but the scene of placodal activity shifts to the rostral margin of the base of the vesicle where diffuse cellular migration is commonly seen, as in Plate 2, fig. I 17. Here the basement membrane is lost and the usual pattern is simply a diffuse outpouring of placodal cells, although distinct spurs or slender files are occasionally found. The placodal cells which detach from this new placodal site are not added to the vestibular part of the pars inferior, but settle in the angle between this and the motor root of the facial nerve.

### *12·0–16·0-mm. stage (26–28 days)*

During the previous stage the apical region of the otic vesicle has steadily expanded in a dorsal direction and by the 12·0-mm. stage has become a broad flattened pouch with curved margins which are beginning to turn laterally. The formation of the posterior semicircular canal is foreshadowed by the appearance of a shallow groove which follows the caudal margin of the otic vesicle a little way from its edge. The anterior canal is similarly outlined at a slightly later stage and the anlage of the horizontal canal develops as a flattened outgrowth which obliquely crosses the middle of the lateral face of the vesicle. With local resorption of parts of the closely apposed and attenuated walls both vertical canals become recognizable features at the 14·0-mm. stage, but the horizontal canal still lags behind in its development (Text-fig. 5).

In the acoustic ganglion the distinction between pars superior and pars inferior is now less marked since the placodal cells of the pars superior have differentiated into young neuroblasts. The whole of the pars superior together with the medial zone of the pars inferior, in which precocious formation of neuroblasts was noted at the 11·0-mm. stage, now constitutes the vestibular ganglion. The superior vestibular nerve has grown into a stout trunk which bifurcates to supply the ampullary swellings at the base of the anterior and horizontal canals. Proximally it is united with the inferior vestibular branch which now supplies several fine nerves to the saccular region of the labyrinth before traversing its medial wall to innervate the ampulla of the posterior canal (Text-fig. 5).

After the 12·0-mm. stage a redistribution of cells in the region of the pars inferior leads to the formation of the cochlear lobe which is a mass of closely packed placodal cells from which the cochlear ganglion is ultimately derived. The development of this ganglion in the sheep appears to be more complicated than the simple division into upper vestibular and lower cochlear ganglia described in the pig by Campenhout (1935*b*) and in the cat by Halley (1955). Cells from two sources participate in the formation of the cochlear lobe. Firstly, there is a gradual decrease in the number of placodal cells found in the medial zone of the pars inferior until by the 12·5-mm. stage only the neuroblasts of the vestibular zone remain. Examination of transverse and coronal sections indicates that this depletion is due not to differentiation of these placodal elements into vestibular neuroblasts, but to their migration to a more ventral position where they mobilize to form the cochlear lobe. Secondly, these residual placodal cells originating from the pars inferior are augmented by continued placodal proliferation along the rostral edge of the vesicle immediately ventral to the superior vestibular nerve. In 12·0- and 13·0-mm. embryos this site shows diffuse cellular migration as well as occasional discrete spurs and files of cells, but this activity ends by the 14·0-mm. stage. The cochlear lobe thus lies between the motor root of the facial nerve and the elongating base of the vesicle which is turning medially as the cochlear bud. Although the greater part of the cochlear lobe consists of placodal cells, a limited number of young neuroblasts are present in the zone adjacent to the cochlear bud. A short nerve outgrowth which connects this zone with the wall of the cochlear bud probably represents the first trace of the distal fibres of the cochlear nerve.



TEXT-FIG. 5. Graphic reconstruction of the otic vesicle, acoustic and facial nerves in a 14·0-mm. embryo. ant. canal, anterior canal; coch. bud, cochlear bud; endo. duct, endolymphatic duct; G. VII, geniculate ganglion; G. VIII coch. lobe, cochlear lobe of placodal cells; hor. canal, horizontal canal; post. canal, posterior canal; VII fac. n., facial nerve; VII gr. sup. pet. n., greater superficial petrosal nerve; VII m. root, motor root of facial nerve; VIII inf. vest. n., inferior vestibular nerve; VIII sup. vest. n., superior vestibular nerve; VIII vest. g., vestibular ganglion.

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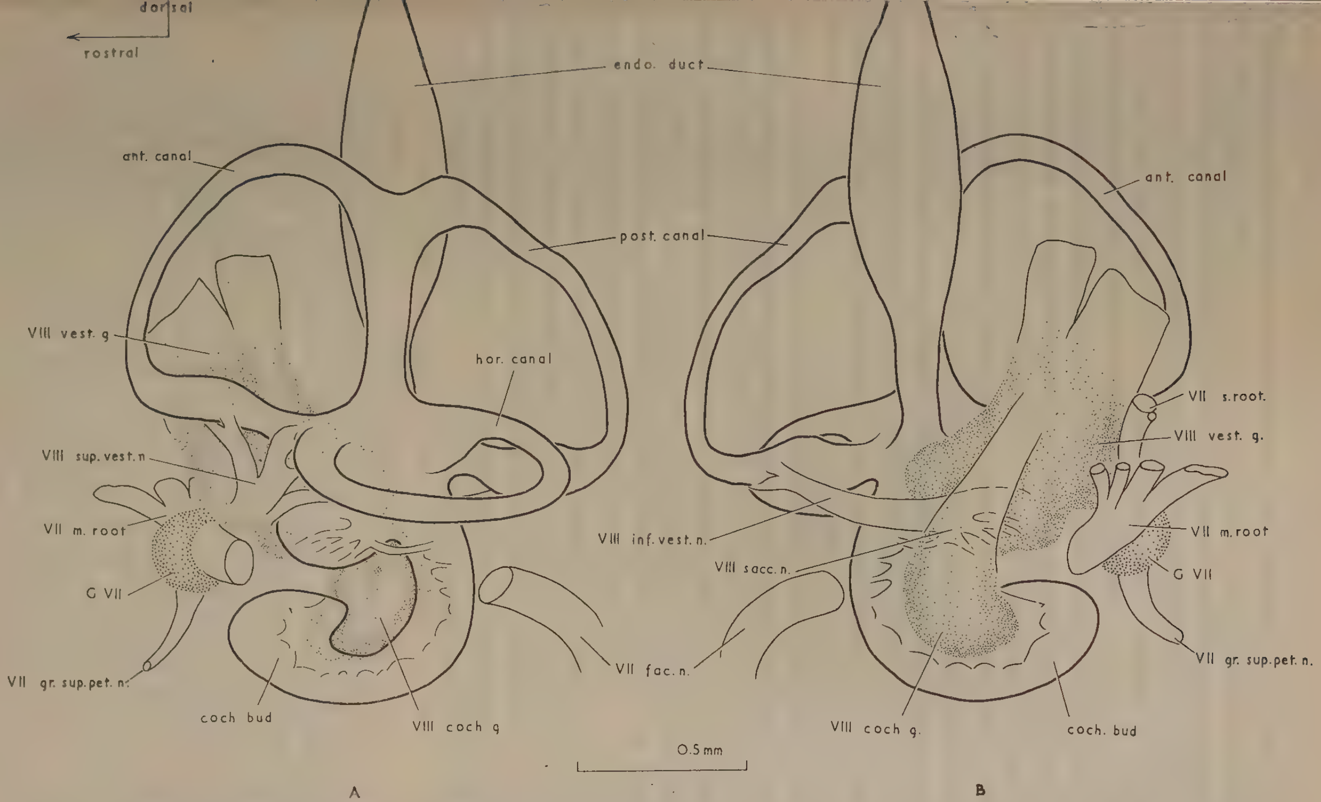

TEX-FIG. 6. Graphic reconstruction of the membranous labyrinth, acoustic and facial nerves in a 26·0-mm. embryo. A: lateral view. B: medial view. ant. canal, anterior canal; coch. bud, cochlear bud; endo. duct, endolymphatic duct; G. VII, geniculate ganglion; hor. canal, horizontal canal; post. canal, posterior canal; VII fac. n., facial nerve; VII gr. sup. pet. n., greater superficial petrosal nerve; VII m. root, motor root of facial nerve; VII s. root, sensory root of facial nerve; VIII coch g., cochlear ganglion; VIII inf. vest. n., inferior vestibular nerve; VIII sacc. n., saccular nerve; VIII sup. vest. n., superior vestibular nerve; VIII vest. g., vestibular ganglion.

*22·0–35·0-mm. embryos (30–35 days)*

As described in the previous stage, the rudiment of the cochlear ganglion is represented by a lobe of placodal cells at the base of the vestibular ganglion, and in order to follow the development of the definitive cochlear ganglion several larger embryos of up to 35·0-mm. G.L. have been examined (Table 1). The reconstruction of a 26·0-mm. sheep embryo (Text-fig. 6) illustrates the advanced differentiation of the membranous labyrinth which in all essential features closely resembles the reconstruction of a 20·0-mm. human embryo prepared by Streeter (1906, Plate 1, figs. l, n). The geniculate ganglion has a markedly pendant shape but still retains a narrow connexion with the vestibular ganglion. As the vestibular neuroblasts are now only slightly smaller than those of the geniculate ganglion, the cytological contrast between these ganglia is considerably diminished. The external morphology of the vestibular portion of the membranous labyrinth now approaches the definitive pattern and the branches of the superior and inferior vestibular nerves can be traced to innervate rudimentary cristae and maculae (Text-fig. 6A).

In comparison with the 14·0-mm. stage the most striking advance has been made in the outgrowth of the cochlear duct which has completed a single spiral turn by the 26·0-mm. stage and encloses in its axis the definitive cochlear ganglion. This ganglion is formed mainly by the migration of the placodal cells of the original cochlear lobe, but there is occasional evidence in the form of attenuated streams of cells projecting from the epithelium of the cochlear duct which suggests a laggard placodal contribution. The majority of the cells of the cochlear ganglion are young neuroblasts, but these are distinctly smaller than those in the vestibular ganglion. Distally, the cochlear ganglion is intimately connected with the inner curvature of the cochlear duct by numerous fine nerve-fibres and proximally the cochlear nerve may be traced across the medial surface of the vestibular ganglion to enter the root of the acoustic nerve (Text-fig. 6B).

## DISCUSSION

The principal conclusion to be drawn from this investigation is that the VII and VIII ganglia originate from specific and initially independent cell masses and not from a common acustico-facial crest primordium. As described elsewhere (Batten, 1957), this crest material receives a contribution from the epibranchial placode and then differentiates into the geniculate ganglion just as Landacre (1932) found in the rat. The acoustic ganglion, however, is essentially of placodal origin and the manner in which its cells proliferate from a dorsolateral placode incorporated in the otic vesicle confirms the findings of Campenhout (1935*b*) in the pig and Groth (1939) in the rabbit. Since the evidence in the sheep contradicts the widely accepted view that the acoustic ganglion arises by subdivision of an acustico-facial primordium, as has been maintained by Adelmann (1925), Bartelmez (1922), Cameron & Milligan (1910), Da Costa (1931), Holmdahl (1934), Kolmer (1928), Schulte & Tilney (1915), Völker (1922), and Weigner (1901), an attempt to reconcile the opposing views might be helpful. A partial explanation lies in the inadequacy of material: many workers have observed the intimate fusion of these ganglia and, lacking or overlooking the transient early stages in which the ganglia are distinctly separate, have been misled into assuming for them a common origin. The comprehensive and influential paper of Adelmann (1925) offers an exception, but it is nevertheless evident from a critical comparison with the parallel work of Landacre (1932) that Adelmann is a convinced opponent of the placodal theory.

In spite of the intimate developmental apposition of the facial and acoustic ganglia the conclusion that they develop from a common mass and, more especially, the identification of this as the 'acustico-facial neural crest' is open to suspicion. Firstly, there is the critical difference in the functional components which these nerves carry. Secondly, a common origin from neural crest is incompatible with Landacre's concept of the specific origin of these functional components (Landacre, 1910*a*, 1911, 1914, 1920; Stone, 1922, 1924, 1928 *a*, *b*, *c*). This concept was founded on fish and amphibian material, but is now considered equally valid for birds and mammals by Ariëns Kappers (1941). According to this concept the acoustic ganglion in the mammal should arise from a dorsolateral placode incorporated in the otic vesicle and not from neural crest. Thirdly, the formation of the acoustic ganglion from neural crest is out of harmony with the pattern of its development in the lower vertebrates. As early as 1907 Brachet showed in the frog that this ganglion arose mainly from cells proliferated from the otic vesicle and later Knouff (1927, 1935) concluded that it was entirely of placodal origin. This finding has been confirmed in other amphibia (Kostir, 1924; Niessing, 1932; Yntema, 1937) and in fish (Landacre, 1910*b*; Veit, 1924). Although it may be criticized as an ill-founded assumption, the view that the acoustic ganglion may arise from neural crest in the mammal is still current in certain textbooks. It is also surprising that the findings of Campenhout and Groth have not attracted the attention they deserve.

The duration of placodal budding from the otic vesicle in the sheep covers the period from the 6·0-mm. (32-somite) to the 14·0-mm. stage and thus coincides approximately with the limits given by Campenhout (1935*b*) for the pig. The earliest anlage of the acoustic ganglion is formed by diffuse cellular migration and is clearly separated from the facial crest by a zone of mesenchyme as in the pig (Campenhout, 1935*b*) and rabbit (Groth, 1939). After the 7·0-mm. stage the evidence for the migration of cells becomes more convincing with the appearance of distinct spurs and files of cells projecting through the basement membrane. Since the facial crest is exclusively concerned in the formation of the geniculate ganglion and the acoustic ganglion is developmentally independent, at any rate initially, there is no justification for the term 'acustico-facial crest' in these species and, as Groth (1939) has already suggested, its use should be rejected. In the literature on human development the use of the term 'acustico-facial ganglion' has been fostered by the reviews of Bartelmez (1922) and Bartelmez & Evans (1926). Bartelmez (1922) agreed that the geniculate portion received a contribution from the epibranchial placode in 4–16-somite embryos, but implied that the remaining portion of this crest provided the acoustic ganglion. Furthermore, he regarded the possibility of the otic plate contributing cells to the ganglion as being equivocal and, in any case, of minimal significance not only in mammals but also in other vertebrates. Recent reports, however, have shown this opinion to be premature, for Rosenbauer (1955) found evidence of a cellular migration from the vesicle in a single 24-somite embryo. Similarly, Politzer (1956), in five human embryos with 26–30 somites, has traced the origin of the rudiment of the acoustic ganglion to diffuse placodal budding from the vesicle, which resembles events in sheep, pig, and rabbit embryos of equivalent stage. The interpretation of this region is undoubtedly more difficult in the human owing to the closer proximity of the facial crest to the otic vesicle. But while Politzer's findings indicate that the early acoustic ganglion is formed from placodal cells in the same way as in the sheep, pig, and rabbit, his assertion that the entire ganglion arises from this source needs to be verified in later stages, especially since Masy (1955) has noted a placodal contribution in a single 9·0-mm. embryo.

In the cat there are also conflicting reports of placodal activity. Schulte & Tilney (1915) consider that the acoustic ganglion arises from the common crest primordium and fail to mention any placodal activity. Halley (1955), however, describes an active cellular migration from the otic vesicle between the 5·0- and 10·0-mm. stages, but owing to the presence of pycnotic debris within the acustico-facial rudiment she was unable to determine whether any neural crest cells survive to become acoustic neuroblasts or whether these are exclusively formed from placodal cells. A careful search for similar pycnotic debris proved negative in the sheep, and the ultimate differentiation of the placodal cells into acoustic neuroblasts can be traced without this complication. Apparently the 'acustico-facial crest' makes contact with the vesicle rather earlier in the cat than in other species, but it would be interesting to know if there is any diffuse cellular migration before this fusion occurs, since it is at this stage that the independent acoustic ganglion first appears in the sheep, pig, and rabbit.

After the 8·0-mm. stage in the sheep the detachment of placodal spurs becomes more vigorous and with the addition of these cells the rapidly enlarging acoustic ganglion becomes closely apposed to the geniculate ganglion to establish the so-called acustico-facial complex. The intimate association of these ganglia has often been misinterpreted as evidence of their common origin. But since these ganglia are developmentally independent at earlier stages and arise from specific anlagen in the sheep, pig (Campenhout, 1935*b*), rabbit (Groth, 1939), and human (Politzer, 1956), their association must be regarded as a secondary event caused by the encroachment of the acoustic upon the geniculate ganglion. This interpretation returns to Streeter's contention that the relationship involves contact rather than fusion with implied developmental significance (Streeter, 1906). In spite of their apposition the two ganglia may still be distinguished by cytological differences which resemble those described in the pig and rabbit, and provide a further argument against the view that these ganglia share a common derivation. In the latter case the two ganglia would show a uniform level of cytological differentiation. But their cytological disparity is caused by the earlier neuroblastic differentiation of the geniculate ganglion which exists as a crest rudiment for some time before there is any indication of the formation of an acoustic ganglion.

The morphological transformation of the enlarging acoustic ganglion into pars superior and pars inferior and the fate of these parts in the sheep closely follows the account given by Streeter (1906) for the human. In the sheep, however, this early subdivision does not correspond exactly with the later differentiation into the definitive vestibular and cochlear ganglia as reported for the pig by Lewis (1902, 1906) and Campenhout (1935*b*) and for the cat by Halley (1955). Moreover, the earliest evidence of the differentiation of placodal cells into acoustic neuroblasts is localized in the lateral zone of the pars inferior and coincides with the outgrowth of the inferior vestibular nerve. In this respect the sheep differs strongly from the pig, for Campenhout describes a cytological subdivision into an upper zone of large pale staining cells (presumably neuroblasts) and a lower zone of small deeply staining cells identical with migrating placodal elements. The definitive vestibular ganglion in the sheep arises from the same material as in the human: it includes not only the pars superior, as Lewis, Campenhout, and Halley envisage, but also the lateral zone of the pars inferior in which the earliest formation of neuroblasts is seen. The remaining medial zone of the pars inferior migrates ventrally, as in the human, to form the rudiment of the cochlear ganglion, but in the sheep this cochlear lobe is augmented by a placodal contribution from the base of the vesicle during the 11·0 14·0-mm. period. There is also some evidence of a limited diffuse migration from the walls of the cochlear bud which has not previously been reported in other species. Thus the formation of the cochlear ganglion in the sheep appears to be more complicated than the simple transformation of the entire pars inferior as envisaged by Lewis (1902, 1906) and Halley (1955). Both in the time of its appearance as a distinct entity and in the differentiation of its placodal cells into neuroblasts, the cochlear ganglion lags behind the vestibular ganglion in the same way as Streeter (1906) found in the human. This retarded development contrasts sharply with the conclusion reached by Campenhout (1935*b*) that the cochlear ganglion develops before the vestibular in the pig. In both the human and the sheep the first neuroblasts appearing in the pars inferior relate to the vestibular ganglion and, if this is so also in the pig, it would then be possible to follow Campenhout's interpretation only by assuming that he may have identified these cells as representing part of the cochlear ganglion, especially since he considers the latter to arise from the entire pars inferior.

Although it would be an unlikely event during the early stages when the primordia are separate the participation of cells of neural crest origin in the formation of the acoustic ganglion becomes theoretically possible after their secondary fusion. Thus, postulating a definite contribution from the facial rudiment to the developing acoustic ganglion, one would expect in the latter a mixture of placodal cells and young neuroblasts equivalent to those of the geniculate ganglion. In the present material, however, the acoustic ganglion arises from an unmixed mass of placodal cells and even the earliest vestibular neuroblasts are of significantly younger stage than those within the geniculate ganglion. While it is improbable that any acoustic neuroblasts originate from the facial crest the possibility that the facial root might provide sheath cells cannot be excluded. Analysis of the development of the acoustic ganglion in the sheep strongly supports the view given by Campenhout for the pig that, if it occurs at all, the contribution provided by the facial crest is negligible. In this respect the acoustic ganglion can be distinguished from the other cranial ganglia as being the only example which is exclusively of placodal origin and in which the neuroblastic differentiation of the placodal cells can be followed without the complication of their mixing with crest elements. The fact that these acoustic placodal cells become neuroblasts and sheath cells provides further support for the view that the cells derived from the trigeminal and epibranchial placodes in the mammal may also share this potentiality.

## SUMMARY

1. The acoustic ganglion arises from placodal cells which detach from the otic vesicle between the 6·0-mm. and 14·0-mm. stages. The accepted view that it develops from a common 'acustico-facial' neural crest is rejected.

2. The initial rudiment of the acoustic ganglion is a mass of detached placodal cells which lies at the base of the vesicle and is distinctly separated from the facial crest by a zone of mesenchyme.

3. After the 8·0-mm. stage the detachment and migration of placodal cells becomes more vigorous and the enlarging acoustic ganglion becomes secondarily apposed to the geniculate ganglion. Nevertheless, these ganglia retain their separate identity through cytological differences which rest on the fact that the young neuroblasts are already present in the geniculate ganglion before a distinct acoustic rudiment is evident. This contrast wanes gradually after the 11·0-mm. stage when the placodal cells begin to differentiate into acoustic neuroblasts.

4. It is concluded that the acoustic ganglion arises exclusively from placodal cells and the participation of facial crest cells in the root portion, if it occurs at all, is probably negligible.

5. The definitive vestibular ganglion is derived from the pars superior together with the lateral zone of the pars inferior in which the earliest neuroblasts appear.

6. The definitive cochlear ganglion begins to develop rather later and arises from the remaining medial zone of the pars inferior with the addition of further diffuse placodal contributions from the base of the vesicle.

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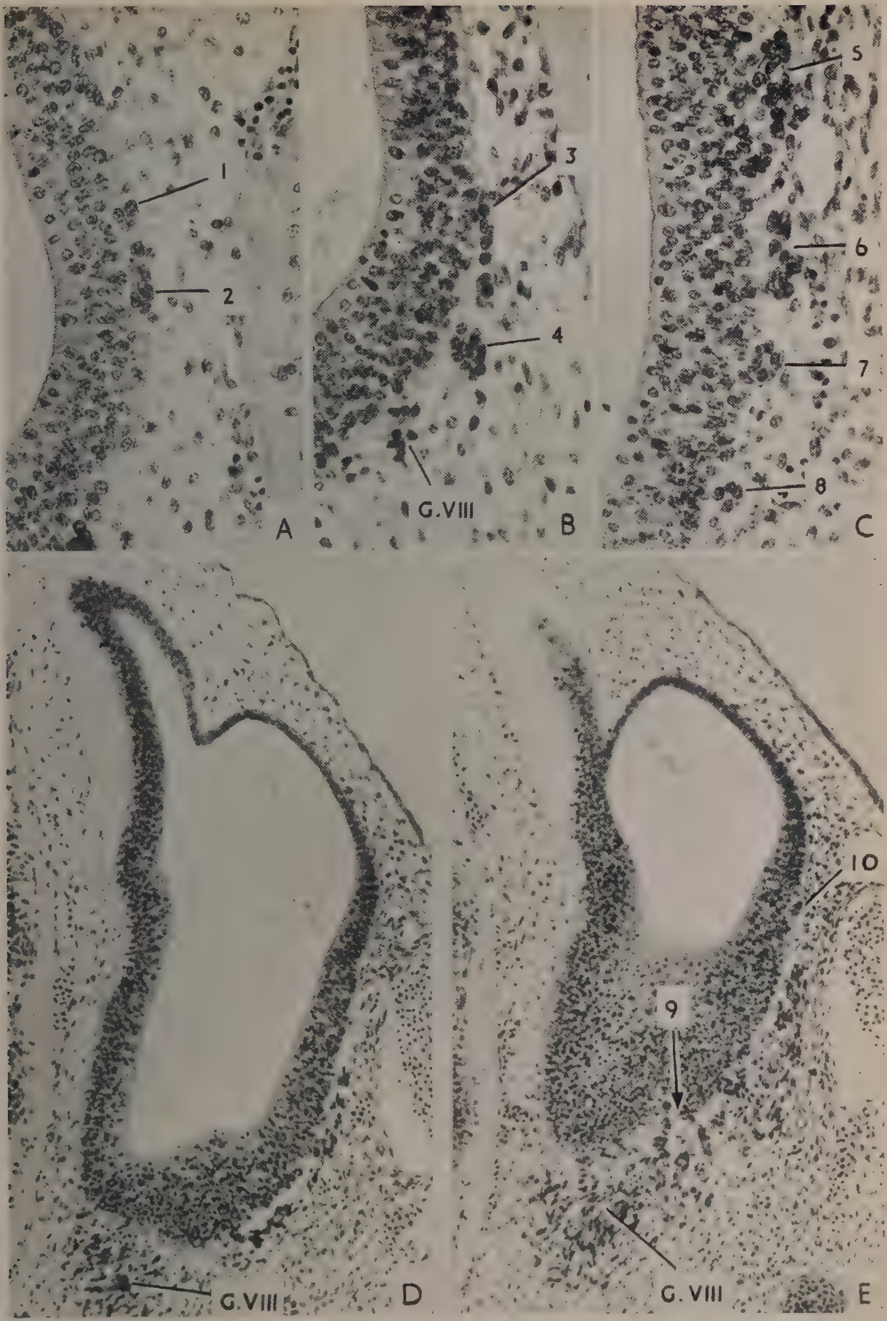
## EXPLANATION OF PLATES

### PLATE 1

FIG. A. Transverse section of a 7·2-mm. sheep embryo showing two early placodal buds (1 and 2) on the medial wall of the otic vesicle. Both buds appear to be sliding ventrally between the parent epithelium and the basement membrane. ×300.

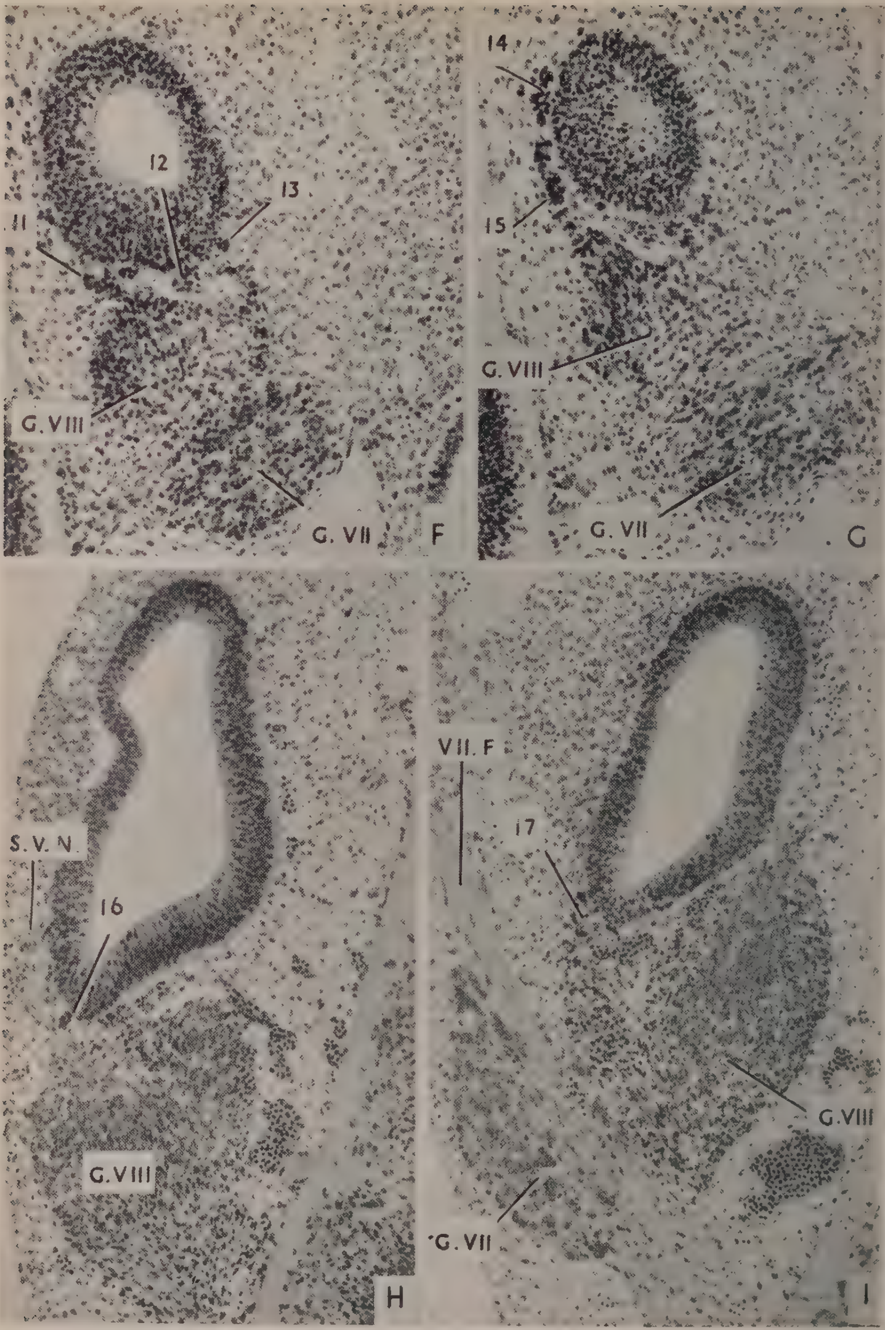
FIG. B. Three placodal spurs associated with the rostro-medial wall of the otic vesicle in an 8·0-mm. sheep embryo. Spur 3 is still enclosed by a tubular evagination of the basement membrane, but spur 4 is completely detached from the epithelium. Immediately below lies the diffuse tip of the mass of detached placodal cells which constitute the rudiment of the acoustic ganglion (G. VIII). ×300.

FIG. C. Transverse section of an 8·2-mm. sheep embryo showing the lateral wall of the otic vesicle with four groups of placodal cells apparently migrating towards the acoustic ganglion.



E. H. BATTEN

*Plate 1*



E. H. BATTEN

*Plate 2*

The upper two spurs (5 and 6) are already free of the otic epithelium. Of the lower spurs, 7 consists of a cluster of cells probably about to detach and 8 is a small free spur which is attached to the otic epithelium in the previous section. × 300.

Fig. D. Coronal section through the cranial third of the otic vesicle in an 8·2-mm. sheep embryo. Note the groups of small deeply staining placodal cells apparently migrating over the lateral face of the vesicle towards the rudiment of the acoustic ganglion (G. VIII). × 130.

Fig. E. Coronal section through the same 8·2-mm. embryo as Fig. D, but passing through the cranial edge of the otic vesicle. The lateral groups of placodal cells have almost reached the acoustic ganglion and make contact with it in the next section. The arrow (9) indicates two spurs of placodal cells which are presumed to be streaming into the ganglion. A small bud of placodal cells probably about to detach from the lateral wall of the vesicle is visible at 10. × 130.

## Plate 2

Fig. F. Transverse section through the ventral region of the otic vesicle in an 8·0-mm. sheep embryo showing three spurs of placodal cells (11, 12, and 13). The cells of the largest spur (11) are presumed to be migrating into the acoustic ganglion. Observe the cytological distinction between the placodal cells of the acoustic ganglion (G. VIII) and the young neuroblasts of the geniculate ganglion (G. VII). × 130.

Fig. G. Transverse section through the same 8·0-mm. embryo as Fig. F, but at a more ventral level. The two large placodal streams (14 and 15) can be traced into the acoustic ganglion in later sections. × 130.

Fig. H. Transverse section through the middle level of the otic vesicle in an 11·0-mm. sheep embryo showing the pars superior and root portion of the acoustic ganglion. The indentation on the lateral wall of the vesicle foreshadows the development of the posterior canal. The superior vestibular nerve (S.V.N.) is faintly visible along the lateral wall of the vesicle with a small placodal bud (16) immediately below it. The small bundle entering the medulla is part of the sensory root of the facial nerve. × 130.

Fig. I. Transverse section through the same 11·0-mm. embryo, but 168 $\mu$ below the level of Fig. H. The pars inferior of the acoustic ganglion sweeps behind the medial wall of the vesicle and is connected with it by the inferior vestibular nerve (I.V.N.). The young vestibular neuroblasts which are differentiating in the lateral zone of the pars inferior contrast sharply with the residual placodal cells of the medial zone which contribute to the cochlear ganglion. The rostral edge of the vesicle shows diffuse placodal migration (17) which is added to the cochlear lobe. The section intercepts the motor root and part of the post-trematic facial branch (VII F) of the facial nerve and illustrates the advanced differentiation of the geniculate neuroblasts (G. VII). × 130.

*(Manuscript received 11 : iv : 58)*

# The Dependence of the Embryonic Inductive Action of HeLa Cells on Their Growth Media

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WITH SIX PLATES

## INTRODUCTION

ALTHOUGH the ability of adult tissues to regulate the differentiation of a competent ectoderm is tissue-specific within certain limits, this inductive capacity appears to be influenced by external factors (see Discussion). In the investigations to be presented here, the effect of various growth media on induction by cells grown *in vitro* was studied, the main purpose being to elucidate the relations between the inductive capacity of the tissues and the external factors. At the same time, it was our intention—by changing the growth medium—to 'fractionate' the inductive capacity of the tissues under known conditions in order to gain further insight into the question of the amount and quality of the inductive agents. Being concerned with malignant cells, this investigation was, in addition, intended as a further link in the chain of experiments by which we have studied the differences between normal and malignant tissues with regard to inductive capacity.

## MATERIAL AND METHODS

The cells employed were HeLa cells, obtained from the State Serum Institute through the courtesy of Dr. Kari Penttinen. With regard to their cultivation the reader is referred to previous papers from that Institute (Penttinen & Saxén, 1956 *a*, *b*). In brief, the method is as follows: the cells are cultured in Roux flasks in a growth medium consisting of 30 per cent. of pooled fresh ('active') human serum and 70 per cent. of Hanks' solution. No embryonic extract was used. This cell strain has been cultured in the laboratory for more than three years, and its mode of growth and morphology have been studied (Penttinen & Saxén, 1956 *a*, *b*; Penttinen *et al.*, 1958). This basic ('control') material was treated by four different methods, which are depicted in Text-fig. 1.

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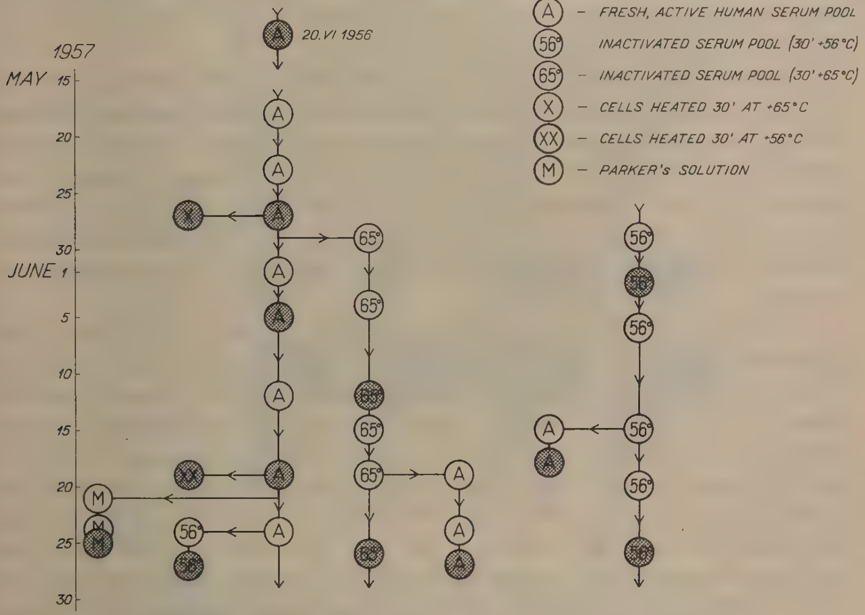
1. Cells grown in active serum were centrifuged, washed as later described and heated in glass tubes on a water bath. Two experiments were performed in this way. In one of them, the cells were heated for 30 minutes at 56° C. (corresponding to the heating of the inactivated serum in which the cell strain is maintained in the laboratory). In the other experiment, a temperature of 65° C. was used. According to previous experience, the latter temperature is sufficient to destroy the mesodermalizing principle in the tissues (Toivonen, 1949).


A – FRESH, ACTIVE HUMAN SERUM POOL
56° INACTIVATED SERUM POOL (30' +56 °C)
65° – INACTIVATED SERUM POOL (30' +65 °C)
X – CELLS HEATED 30' AT +65 °C
XX – CELLS HEATED 30' AT +56 °C
M – PARKER's SOLUTION
20.VI 1956
1957
MAY 15
20
25
30
JUNE 1
5
10
15
20
25
30
A
X
XX
M
56°
65°

TEXT-FIG. 1. Scheme of cultivation of the HeLa cells used in the investigations. Each circle represents a transplantation of cells, and the hatched circles stand for the cultures used in implantation experiments. In these cases the cells were detached and some were used for further cultivation, whilst the greater part was used in induction experiments.

2. In the second series of experiments, the cell strain as routinely maintained in the laboratory was studied. The pooled serum used in routine cultivation of this strain is inactivated by heating on a water bath for 30 minutes at 56° C. (Penttinen & Saxén, 1956*b*). In addition, an experiment was performed in which cells grown in active (i.e. untreated) serum were placed for 3 days in serum inactivated in the manner described. A parallel experiment was performed in which cells continuously grown in inactivated serum were placed for 3 days in active serum.

3. As growth medium a pooled serum was used which had been heated on

a water bath for 30 minutes at 65° C. In the first experiment in this series the cells survived over three passages (21 days), but subsequently exhibited markedly decreased growth. At this stage a proportion of the cells were used in implantation experiments, but since the remainder proved to be incapable of further growth, the former were also regarded as inviable and the results were discarded. The second series of experiments was started on 29 May 1957, and the growth of the cells was satisfactory until autumn 1957, when the whole culture accidentally perished (Penttinen *et al.*, 1958). These cells were used twice in implantation experiments, i.e. after the second (14 days) and fifth (28 days) passages. As in the foregoing series, the cells were transferred to active serum, but owing to technical difficulties the series became too small.

4. In the fourth series of experiments pure Parker's 199 solution was used as growth medium instead of active serum. In these experiments a different technique was used, inasmuch as the cells were not detached from the walls of the flasks and placed in the solution, but instead the serum was poured off and replaced by Parker's solution. After 3 days this growth medium was renewed, and on the next day the cells were used in experiments. Throughout the experiments the growth of the cells was poor, but dead, detached cells were not observed.

All the sera used were obtained from healthy donors of the Finnish Red Cross Blood Bank. The serum pools used in the cultures consisted of 3–10 sera.

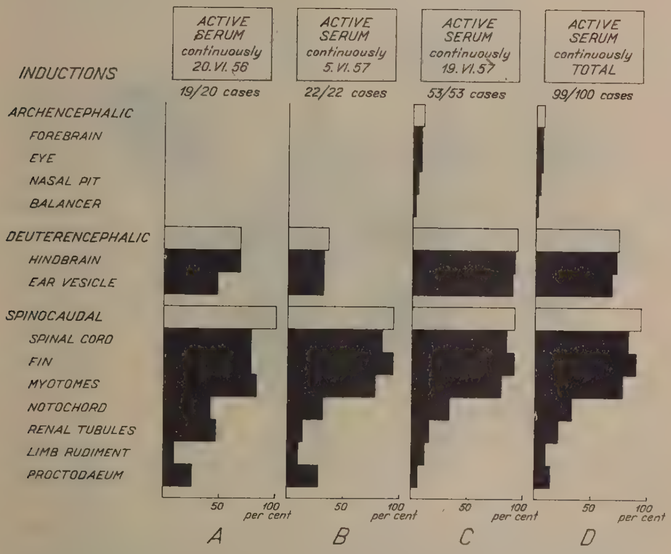
The after-treatment of the cells was the same in all experiments: the cells adhering to the walls of the Roux flasks were mechanically detached without trypsinization (detached cells in the growth medium were not used). The cells were first centrifuged in Corda tubes, and the loose sediment of cells thus obtained was suspended in 10 ml. of sterile saline solution. Centrifugation was then performed in 15 ml. tubes at 20° C. for 20 minutes at 5000 r.p.m. The supernatant fluid was poured off and the cells were again suspended by vigorous shaking in the same amount of fluid and centrifuged. Washing was repeated three times. After the third centrifugation, the fluid was replaced by 70 per cent. alcohol, the suspension was again centrifuged, and the cells were used for implantation within 12 hours.

In the implantation experiments, young *Triturus* gastrulae were used as hosts, and the technique was the same as that described in many previous papers (Toivonen & Saxén, 1955). The cells, which had been kept in alcohol, were washed in Holtfreter-Ringer solution and implanted into the blastocoele, and subsequently the larvae were reared for 12 days. All the larvae which developed normally were fixed, sectioned in complete series and examined under the microscope. The final series included all individuals in which the inductor was found to be in contact with the ectoderm or in which an induction was clearly observable. No toxic effects were demonstrable and the mortality of the hosts was extremely low.

## RESULTS

For the sake of simplicity, all the primary results are presented in one table at the end of this section, but the results of the various experiments, expressed as percentages, are given separately in diagrammatic form in connexion with the respective reports.

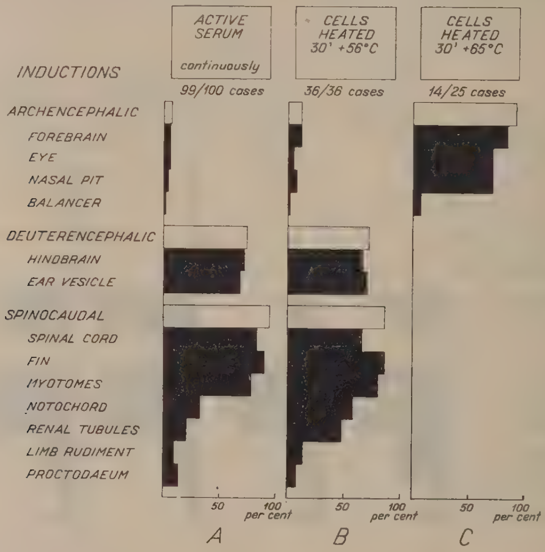
*Cells cultured in pooled active serum.* Control cells from the same line were investigated four times, the first time in 1956 and subsequently in spring 1957 (Text-fig. 2). For technical reasons the third series was too small to be considered alone, but it was included in the final calculations. Thus, the total number of larvae approved was 100, 99 of which showed inductions. The cells proved to be strong inductors, mostly causing a macroscopically discernible tail reaction and a protuberance containing deuterencephalic brain parts (Plate 1). The commonest structures were hindbrain parts, ear vesicles, spinal cord, fin margin, and myotomes. These formations were found in 70–90 per cent. of cases. A notochord, renal tubules, and limb rudiments were more occasional, and archencephalic structures were rare. A purely qualitative evaluation does not afford an adequate picture of the strength of the various types of induction, but, as is seen in the plates, the spinal inductions were as a rule very conspicuous, whilst the archencephalic structures were inconsiderable.



TEXT-FIG. 2. The inductive effect of HeLa cells grown in untreated ('active') serum. *A–C* represent the same line studied at different times, and *D* represents all cases included in the final results. (The numbers denote the positive cases and the total number of larvae.)

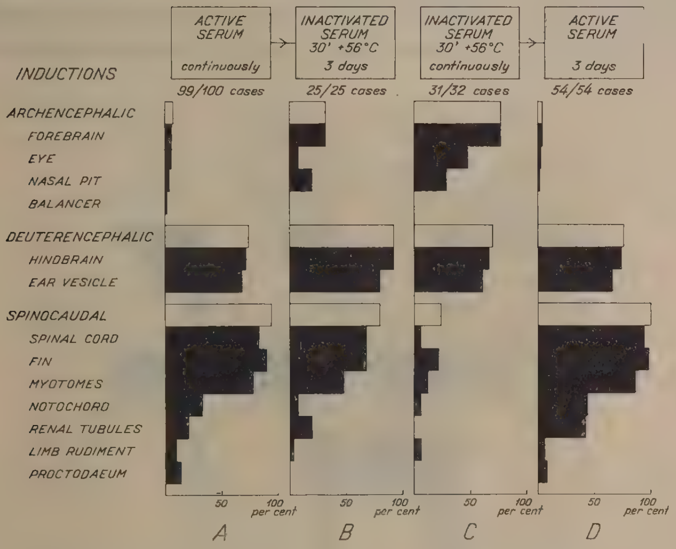
On comparison of three series of experiments performed at different times, no fundamental differences were observable. The last of these series exhibited somewhat more numerous neuralizations than the others, but the mesodermal structures were equally frequent and of the same kind.

*Cells heated at 56° and 65° C.* The purpose being to study the effect of heated serum used as growth medium, it was regarded as necessary to check the influence of the heating process on the cells themselves (i.e. on cells cultured in active, i.e. untreated, serum). As seen in Text-fig. 3 and Plate 3, the two temperatures in question had different effects. Heating for 30 minutes at 56° C. did not influence the inductive capacity of the cells at all, the resultant inductions corresponding completely to those caused by unheated cells. By contrast, after heating for the same length of time at 65° C., spinocaudal and deuterencephalic inductions were entirely lacking. In addition, a general weakening of the inductive capacity of the cells was noted; in many cases no induction resulted, although the implant was clearly in contact with the ectoderm. In the other experiments such negative cases were rare.



TEXT-FIG. 3. The inductive effect, after heating, of cells grown in untreated ('active') serum. Unheated cells were used as controls (Text-fig. 2D).

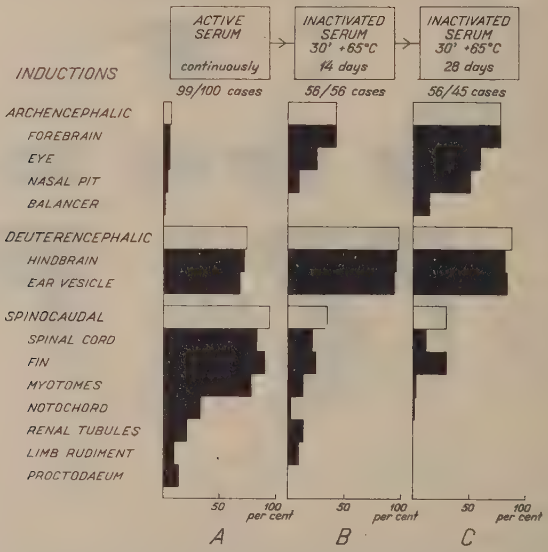
*Cells grown in pooled human serum heated for 30 minutes at 56° C.* First, the laboratory strain continuously cultured for three years in inactivated serum (heated at 56° C.) was investigated (Text-fig. 4C, Plate 4). The cells were investigated twice, but the second experimental series was very meagre. The results show that the inductive capacity of the cells had clearly changed. Inductions were still encountered in almost 100 per cent. of the larvae, but the structures were different. As compared with the controls, spinocaudal formations occurred in markedly decreased numbers, notochord, spinal cord, renal tubules and limb rudiments being observed only in one or two instances, myotomes rarely, and fin margins somewhat more often. It should again be emphasized that the impression conveyed by a purely qualitative enumeration is somewhat misleading. Thus, in the whole series there was not a single instance of an obvious, regular tail reaction, which in the series with cells grown in active serum was the commonest reaction; the myotomes consisted only of some detached fibres, and both spinal cord and notochord appeared as short, thin strings. The deuterencephalic inductions did not show any marked changes as compared with the controls. By contrast, the archencephalic structures (forebrain, eye, nasal pit, balancer) differed greatly from those encountered in the control experiments, being much more frequent and more conspicuous.



TEXT-FIG. 4. The change in inductive effect of HeLa cells grown in heat-inactivated serum (heated for 30 minutes at 56° C.). (*A*) controls ('active' (untreated) pooled serum), (*B*) cultivation for a short period in heat-inactivated serum, (*C*) continuous cultivation (over three years) in heat-inactivated serum. Diagram *D* shows the inductive effect of the cells when, after continuous cultivation in heat-inactivated serum (*C*), they were returned for 3 days to active serum. The inductive effect is exactly the same as that of the cells continuously grown in untreated serum (*A*).

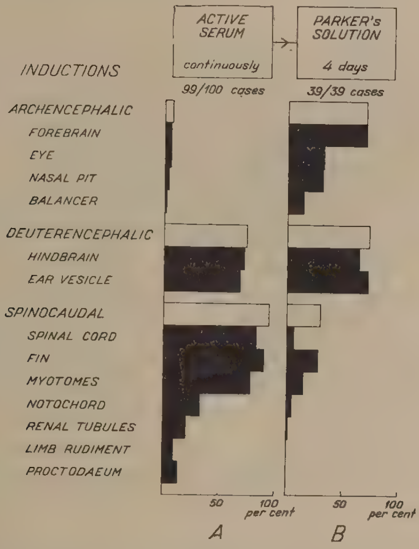
In addition to the cells continuously grown in heat-inactivated serum, cells were studied which were transferred for 3 days from active serum to inactivated serum (heated at 56° C.) (Text-fig. 4B). This series also exhibited differences as compared with the controls, although they were slighter than in the foregoing series. The change in the inductive capacity of the cells showed the same trend, however: the spinal inductions decreased and the archencephalic inductions increased.

For reasons to which we shall return in the Discussion, we wanted to find out whether this change in the inductive capacity of the cells was permanent or dependent on the growth sera. Hence, an experiment was performed in which cells cultured continuously in inactivated serum were returned for 3 days to active serum. When these cells were subsequently used for implantation, inductions developed in 100 per cent. of cases. The resultant structures are presented in Text-fig. 4D. It is seen that within 3 days the inductive capacity of the cells returned completely to 'normal', inasmuch as the resultant inductions corresponded to those induced by cells continuously grown in active serum: the archencephalic tendency disappeared almost completely and regular tails and deuterencephalic brain parts were the commonest structures induced (Plate 5).



Text-fig. 5. The gradual change in the inductive effect of HeLa cells occurring when they are cultivated in heat-inactivated serum (heated for 30 minutes at 65° C.). After 28 days the effect is almost the same as after continuous cultivation in heated serum at 56° C (Text-fig. 4C).

*Cells grown in pooled human serum heated for 30 minutes at 65° C.* We knew from previous investigations that such heating destroys the mesodermalizing principle in the tissues (Toivonen, 1949). Hence it was assumed that in serum thus treated this principle would not be present to any notable degree, and that it would also be lacking in the cells cultured in such serum, if the properties of the cells are dependent on the growth medium. The cells were used for implantation after 14 and 28 days' culture (Text-fig. 5 B, C; Plate 2). On examination of the resultant inductions, it was found that the weakening of the mesodermalizing factor observed in the experiments described in the foregoing section was also demonstrable in these experiments. Even after 14 days, spinocaudal structures had developed in markedly decreased numbers, and after 28 days, an occasionally occurring small fin margin and a short spinal cord were almost the only spinocaudal structures formed. Simultaneously there was an increase in archencephalic structures, which after 14 days had developed in 43 per cent. and after 28 days in 77 per cent. of the larvae against 6 per cent. in the controls. With regard to the occurrence of deuterencephalic inductions, the differences were negligible. As in the foregoing series, the cells grown in heated serum were returned to active serum. The series of implantation experiments then performed was too small, however, to allow of any conclusions, but a mesodermal 'reactivation' was observable, as in the foregoing series.



TEXT-FIG. 6. The change in inductive effect of HeLa cells grown in protein-free growth medium (Parker's 199 solution).

*Cells grown in protein-free solution.* Two series of experiments were performed, but in the first the cells were not viable at the time when the implantation experiments were made. In the second, the cells lived for 4 days in pure

Parker's solution, which was changed on the third day. At the end of this period the cells were still microscopically normal and adherent to the walls of the culture flask. The inductive capacity of the cells (Text-fig. 6 B, C; Plate 6) was found to be almost equal to that of the cells grown continuously in serum heated at 56° C. or for 28 days in serum heated at 65° C., spinal structures occurring

TABLE 1

| *Implantation tissue (HeLa cells grown in different culture media)* *(The data given refer to Text-fig. 1)* | *Number of larvae examined* | *Number of larvae with induced structures* | *Induced structures* | | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | *Head formations* | | | | | | *Trunk and tail formations* | | | | | | |
| | | | *Epidermal and neural* | | | | | | | *Mesodermal* | | | | | |
| | | | *Forebrain* | *Eye* | *Nasal pit* | *Balancer* | *Hindbrain* | *Ear vesicle* | *Spinal cord* | *Fin* | *Myotomes* | *Notochord* | *Renal tubules* | *Limb rudiment* | *Proctodaeum* |
| *Cells grown in active pooled human serum:* | | | | | | | | | | | | | | | |
| 1. 20.6.1956 . . . | 20 | 19 | 0 | 0 | 0 | 0 | 13 | 9 | 15 | 15 | 16 | 8 | 9 | 2 | 5 |
| 2. 27.5.1957 . . . | 5 | 5 | 1 | 1 | 1 | 0 | 3 | 4 | 4 | 5 | 2 | 0 | 1 | 0 | 1 |
| 3. 5.6.1957 . . . | 22 | 22 | 0 | 0 | 0 | 0 | 7 | 7 | 19 | 21 | 17 | 7 | 3 | 2 | 6 |
| 4. 19.6.1957 . . . | 53 | 53 | 5 | 5 | 3 | 1 | 49 | 48 | 45 | 49 | 43 | 18 | 8 | 6 | 3 |
| Total . . . | 100 | 99 | 6 | 6 | 4 | 1 | 72 | 68 | 83 | 90 | 78 | 33 | 21 | 10 | 15 |
| *Heated cells:* | | | | | | | | | | | | | | | |
| 30′ at 56° 19.6.1957 . | 36 | 36 | 4 | 2 | 3 | 1 | 23 | 25 | 24 | 31 | 28 | 21 | 17 | 5 | 3 |
| 30′ at 65° 27.5.1957 . | 25 | 14 | 12 | 10 | 10 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cells grown in pooled serum heated 30′ at 56°:* | | | | | | | | | | | | | | | |
| 3 days 27.6.1957 . . | 25 | 25 | 8 | 2 | 5 | 0 | 23 | 20 | 17 | 17 | 12 | 2 | 5 | 1 | 0 |
| Continuously | | | | | | | | | | | | | | | |
| 1. 2.6.1957 . . | 29 | 28 | 21 | 13 | 9 | 4 | 20 | 18 | 2 | 7 | 4 | 0 | 2 | 2 | 0 |
| 2. 26.6.1957 . . | 3 | 3 | 3 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total . . . | 32 | 31 | 24 | 15 | 9 | 4 | 20 | 19 | 2 | 7 | 4 | 0 | 2 | 2 | 0 |
| Transferred to untreated pooled serum for 3 days: 18.6.1957 . . . | 54 | 54 | 3 | 2 | 1 | 0 | 41 | 36 | 51 | 53 | 47 | 24 | 23 | 3 | 4 |
| *Cells grown in pooled serum heated 30′ at 65°:* | | | | | | | | | | | | | | | |
| 14 days 12.6.1957 . . | 56 | 56 | 24 | 15 | 11 | 1 | 54 | 53 | 12 | 14 | 9 | 2 | 9 | 6 | 0 |
| 28 days 26.6.1957 . . | 45 | 45 | 35 | 27 | 23 | 6 | 37 | 38 | 6 | 14 | 2 | 1 | 0 | 0 | 0 |
| *Cells grown in serum-free culture media:* | | | | | | | | | | | | | | | |
| 4 days 25.6.1957 . . | 39 | 39 | 27 | 12 | 12 | 5 | 25 | 29 | 3 | 11 | 5 | 1 | 2 | 0 | 0 |

in 30 per cent. of cases (a small and irregular fin margin being the commonest induction), deuterencephalic in 75 per cent., and archencephalic in 70 per cent. Typical tails with two fins were not observed, but some irregular, mesenchymal folds with a fin margin occurred, which sometimes contained small myotomes and, occasionally, a short, narrow spinal cord.

## DISCUSSION

Previous experiments with heterogeneous inductors have shown that the tissues of an adult individual have a qualitatively and quantitatively typical inductive effect when implanted into young gastrulae of the newt (Chuang, 1938, 1939; Toivonen, 1938, 1940, 1953; Engländer, Johnen, & Vahs, 1953; Yamada & Takata, 1955; Engländer & Johnen, 1957). We have advanced the hypothesis (Toivonen & Saxén, 1955) that the nature of the inductive effect of a heterogeneous inductor is dependent on the combination of inductive agents which it contains. We believe that two active principles, namely, a neuralizing (N) and a mesodermalizing (M) principle, are responsible for the inductive effect. If a tissue contains only N-principle in active form, it induces the competent tissue to develop into archencephalic structures, i.e. forebrain and/or its derivatives. If, in addition to the N-principle, a relatively weak M-principle is present, the competent tissue differentiates into deuterencephalic structures, i.e. hindbrain and its derivatives. If, on the other hand, the inductor tissue contains a strong M-principle in addition to the N-principle, spinocaudal structures (spinal cord, notochord, myotomes, renal tubules, limb rudiments, &c.) will be induced.

It has proved possible to modify the original inductive effect of the tissues by treating them *in vitro*. In our opinion the above-mentioned hypothesis explains all the relevant results on the assumption that one or other of the active principles is weakened or completely inactivated. Thus, when the influence of the M-principle is decreased, the inductive action becomes more cranial in nature, and when the N-principle is weakened, the effect of the inductor will be more caudal than before.

The M-principle can be completely inactivated in a tissue by heating the latter for a short time in water at a temperature of at least 65°–70° C. (Chuang, 1940; Toivonen, 1949, 1954). Irrespective of what structures were previously induced by the tissue, it changes into a purely archencephalic inductor and at the same time its neuralizing capacity seems to be strengthened. We do not believe, however, that this is due to a change of the M-principle into the N-principle as a result of the heat treatment, as Chuang (1940) and Yamada & Takata (1955) have ventured to suggest. Their view is invalidated by a comparison of the effect of heat treatment on renal tissue and bone marrow tissue. After alcohol fixation the former is a strong deuterencephalic-spinocaudal inductor, which, in addition to hindbrain and spinal cord, induces mesodermal trunk and tail structures. Similarly treated, the latter tissue is an almost pure, very strong mesodermal inductor, which induces mesodermal trunk and tail structures and sometimes, in addition, a thin spinal cord in the tip of the tail. After heat treatment, renal tissue regularly induces forebrain and its derivatives (Toivonen, 1949), whilst the bone marrow causes, at the utmost, a weak archencephalic reaction consisting of epidermal derivatives typical of this region, for instance lenses (Toivonen, 1954). Originally both tissues contained a strong M-principle. If, during heat treatment, the latter changed into N-principle, as suggested by the above-mentioned writers, the two tissues in question would, after heat treatment for a short period, be equally strong inductors of archencephalic structures.

In a recent experiment, however, Yamada (1958) has observed a relatively strong archencephalic action of bone marrow after brief heat treatment. After 60 seconds' steam treatment the bone marrow lost its mesodermalizing action completely, but meanwhile an archencephalic action appeared, which, however, was destroyed almost completely by 150 seconds' steam treatment. Thus, the inductive action of tissue heated for 150 seconds was similar to that of the bone marrow of the earlier experiments referred to above (Toivonen, 1954), in which the tissue was heated for a longer time in hot water. These new results have been interpreted by the author as corroborating his earlier theory of the transformation of the inductive agents into each other. In this respect the results of Yamada contradict our opinion and this disagreement needs further study.

The M-principle is also destroyed in the tissues by formol treatment (Kuusi, 1951) or by prolonged (1–4 weeks) alcohol treatment (Engländer, Johnen, & Vahs, 1953; Engländer & Johnen, 1957). After such treatment the inductive effect of guinea-pig kidney, for instance, changes, the spinocaudal effect almost completely disappearing and the archencephalic effect being strengthened.

The N-principle of an inductor tissue is weakened, for instance, by extraction with petrol ether (Toivonen, 1949). After such treatment guinea-pig liver tissue, the primary effect of which is mainly the induction of archencephalic structures, but which causes deuterencephalic inductions in about 40 per cent. of cases (Toivonen, 1951), becomes almost inactive, although it still occasionally induces a weak tail reaction (fin with mesenchyme). A corresponding weakening of the N-principle after extraction with petrol ether is observable in renal tissue, which when thus treated no longer induces deuterencephalic structures, even the induction of spinal cord being less frequent and less prominent than before, whilst the mesodermalizing action seems to have become stronger (Toivonen, 1949). The N-principle may also be destroyed or weakened by dialyzing minced tissue in water. Liver tissue, for instance, when thus treated, even assumes a weakly spinalizing character (Toivonen, 1951).

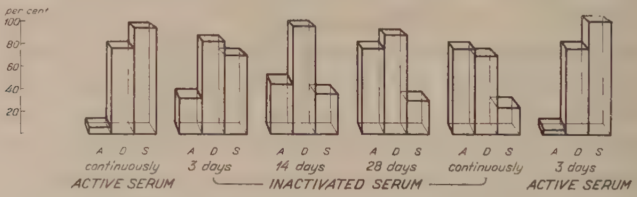
As already mentioned in the Introduction, it is also possible to some extent to influence the inductive effect of the tissues *in vivo*. It is known that if the experimental animals are starved before the inductor tissues are taken, the nature of the inductive effect changes, in that structures of a more cranial nature than before are induced (Toivonen, 1951; Engländer & Johnen, 1957).

If a tissue used in implantation experiments is originally a combined neuralizing-mesodermalizing inductor, it has been observed that after starvation the spinocaudal structures induced are weaker, whilst cranial brain parts and their derivatives are more conspicuous and more frequent. If, on the other hand, the tissue was originally a weak inductor of spinocaudal structures, no such strengthening of the archencephalic effect is discernible (Engländer & Johnen, 1957). It appears that after starvation the animal tissues become poorer in M-principle, and the immediate result of this would be, according to our hypothesis, a strengthening of the effect of the N-principle, since this principle will act in combination with a weaker M-principle than before. In these experiments, at least, starvation did not cause any weakening of the N-principle. On the contrary, a strengthening of its effect occurred, which may be interpreted as indirectly due to a weakening of the M-principle. The obvious stability of the N-principle in the tissues was also demonstrated in our experiments with regenerating rat liver (Saxén & Toivonen, 1957). Although after hepatectomy the actively regenerating liver tissue differs greatly, chemically, from the normal liver tissue (e.g. Saxén & Toivonen, 1957), no differences were demonstrable with regard to the inductive effect, which was purely archencephalic.

Our experiments with normal and leukaemic bone-marrow have also shown that the M-principle in the tissues is not stable. Normal rat bone-marrow is a strong mesodermalizing inductor, but when bone-marrow from a leukaemic individual was used, such an inductive effect was almost completely lacking (Saxén & Toivonen, 1956; Toivonen & Saxén, 1957).

With regard to inductive capacity, the HeLa cells grown in active serum used in the experiments described in the foregoing strongly resembled guinea-pig kidney. The percentage figures expressing the frequency of the archencephalic, deuterencephalic, and spinocaudal structures induced by guinea-pig kidney are 3, 56, and 89 (Engländer & Johnen, 1957). In the present experiments the corresponding figures were 7, 75, and 94. Heating of the HeLa cells for 30 minutes at 65° C. on a water-bath changed their effect into a purely archencephalic one. Thus, their M-principle was found to be thermolabile, just as is that of renal tissue. In conclusion, HeLa cells grown in active serum can be regarded as combined inductors resembling renal tissue, the nature of their inductive effect being determined by the combined influence of a strong M-principle and an N-principle.

When the change in inductive effect caused by changes in the culture conditions was chosen as the subject of study, this afforded a better approach than before to the problem of whether and to what extent the inductive capacity of the tissues is dependent on external and varying factors. It was found that in cells grown in heated serum or in a protein-free growth medium the inductive effect was changed in the same direction as the inductive effect of renal tissue was changed by starvation (Engländer & Johnen, 1957). In both cases, the M-principle was markedly weakened with a resultant cranialization of the neural structures induced. This increase in archencephalic structures caused by the weakening of the M-principle is clearly demonstrated in Text-fig. 7, where the results obtained with sera heated at 56° and 65° are combined, and the length of the culture period is plotted against changes in the inductive capacity of the cells. The figure shows that the M-principle gradually weakened during the first 3 weeks of culture, and simultaneously archencephalic structures gradually became more common. In this respect there is a close parallelism between the starvation experiments with guinea-pig renal tissue made by Engländer & Johnen (1957) and our own experiments. Since it may be concluded that in our experiments the heating of the serum affected the thermolabile M-principle or its precursors, it can be claimed with greater confidence than before that the M-principle in the tissues is dependent on factors which the tissue continuously receives from outside. Thus, the M-principle appears not, or at least not entirely, to belong to the tissue structures proper, but the amount in which it is present seems to depend on the quality of the nutritional fluid bathing the cells or the tissues. The fact that the various tissues of one and the same individual have a different tissue-specific inductive effect seems to suggest that the various tissues have a different affinity for the factors responsible for the inductive process, or their precursors.



TEXT-FIG. 7. The change in inductive effect of HeLa cells during cultivation in heat-inactivated serum. In the diagram the results obtained with sera heated at 56° and 65° C. have been combined. In addition, the result after return to untreated ('active') serum is shown. *A*. archencephalic inductions. *D*. deuterencephalic inductions. *S*. spinocaudal inductions. The diagram shows the decrease in spinocaudal structures and the increase in archencephalic structures as a function of time.

Our views regarding the role of the growth medium and our hypothesis regarding the occurrence of the two active principles are substantiated by the experiment in which cells grown in inactivated serum were returned for a short time to active serum (Text-fig. 4 C, D). It was found that the previously weakened M-principle was rapidly restored in the cells and that, in accordance with our gradient hypothesis, archencephalic structures simultaneously disappeared almost completely. It does not seem in the least probable that transference of the cells to a richer growth medium would destroy the factor in the cells inducing archencephalic structures, but it appears obvious that a strengthening of the

M-principle changed the N/M balance and at the same time caudalized the neural structures.

A noteworthy observation in the present investigation was that heating clearly affected the factors constituting the M-principle of the cells and of the serum differently. Heating of the cells for 30 minutes at 65° C. destroyed their M-principle, whereas heating for the same length of time at 56° C. did not alter their mesodermalizing capacity at all. When serum was heated at these two temperatures, by contrast, the inductive capacity of the cells grown in these sera was equally influenced. The explanation of this seems to be either that the factor responsible for the M-principle of the serum is not entirely the same as the final M-principle of the cells, or that the proteins in a solution are generally more labile than the proteins in tissues.

Since it is obvious that the HeLa cell strains cultured in different laboratories have, during the course of years, to some extent developed by selection in different directions (Chang, 1957), it might be suggested that cultivation in poorer serum and in serum-free solution would bring about a change in the inductive effect of the cells, which would be due to the fact that only cells of a certain genotype, to which the cells originally containing M-principle do not belong, would endure these extreme conditions, and that the cells containing M-principle would therefore gradually disappear from the culture. The change in the inductive capacity of the HeLa cells in exactly the same direction in heated serum and in Parker's solution would seem, then, to indicate that a similar selection occurred in the two instances. In Parker's solution the proliferation of the cells very soon came to a standstill. Hence, a very short time was available for the selection, but theoretically it may, of course, have occurred. In addition, it should be noted that the M-principle was more rapidly affected in the experiments where Parker's solution was used as a culture medium than in those where the cells were grown in inactivated serum, although the proliferation of the cells and thus also the selection, if there was any, were slower in the former. Furthermore, the experiments in which we returned cells, cultured for years in inactivated serum, to active serum showed that the results can hardly be attributed to selection. Immediately after this return, the cells exhibited exactly the same inductive effect as cells continuously grown in active serum.

Since, on the basis of all the facts enumerated above, it is obvious that the M-principle in the cells is essentially dependent on factors in the growth medium, the question arises as to whether this factor or these factors are present as such in the tissue fluids, or whether the cells only require some precursor(s) for their synthesis. In numerous previous investigations it has been demonstrated that the M-principle is associated with the protein fractions of the tissues (Chuang, 1940; Lehmann, 1945; Kuusi, 1951, 1957; Toivonen, 1951; Vainio, 1957; Yamada, 1958), but this observation does not rule out the possibility that the cells absorb this factor from the serum. It is obvious that in tissue culture the cells are, at least in part, capable of utilizing the proteins of the growth medium as such (Francis & Winnick, 1953; Kent & Gey, 1957). In addition, we have been able to show that both guinea-pig serum and human serum contain an active, mesodermalizing factor, although it is weak or easily destroyed in experiments (Vainio, Toivonen, & Saxén, 1958). On the other hand, it is also possible that the cells require from external sources only a part of the M-principle or a precursor of it, synthesis proper occurring in the cell. This view would, perhaps, obtain some support from the difference in thermolability between the M-principle of the serum and the cells, discussed above, and from our observation that continuous cultivation in inactivated serum does not completely destroy the mesodermalizing capacity of the cells. It should be recalled that in experiments with such cells both a definite deuterencephalic inductive effect and a weak mesodermalizing capacity were demonstrated. A possible explanation of this would be that the cells are to some extent capable of using factors present in inactivated serum and of synthesizing from these an active M-principle. This hypothesis is also corroborated by our experiments with cells grown in Parker's solution, in which it was found that the mesodermalizing capacity disappeared from the cells much more rapidly than in the experiments where heated serum was used as a growth medium. The conclusion may, perhaps, be drawn that even heated serum contains factors which are lacking in Parker's solution, which the cells grown in such a serum can to some extent use for the synthesis of the M-factor. This would explain the slower loss of this factor as compared with the results with pure Parker's solution.

## SUMMARY

HeLa cells which had been cultured in various growth media were used as implantation material in induction experiments, the object of which was to find out to what extent the inductive capacity of the cells is dependent on the media.

1. HeLa cells grown in untreated ('active') pooled human serum proved to be strong deuterencephalic-spinocaudal inductors. The inductive capacity was the same in cells grown in different pooled sera.
2. Heating of cells grown in active serum for 30 minutes at 56° C. did not influence their inductive capacity. By contrast, heating for 30 minutes at 65° C. completely destroyed the deuterencephalic and spinocaudal inductive effects, but subsequently the cells still induced archencephalic structures.
3. Continuous cultivation in serum heated at 56° C. almost completely destroyed the spinocaudal inductive effect of the cells. Simultaneously, the archencephalic effect was strengthened. Cultivation under the same conditions for a short time (3 days) caused a similar, though slighter, change. When some of the cells continuously grown in heat-inactivated serum were returned for 3 days to active serum, their inductive capacity was found to be almost the same as that of cells continuously grown in active serum.
4. Cultivation in serum heated for 30 minutes at 65° C. markedly weakened

the spinocaudal inductive capacity of the cells, although the latter still grew normally. Even after cultivation for 14 days this weakening was clearly observable, and at the same time there was an increase in the number of archencephalic structures. After 28 days' cultivation the cells exhibited the same inductive effect as cells continuously grown in serum inactivated at 56° C.

5. Cultivation in serum-free Parker's 199 solution rapidly weakened the spinocaudal inductive capacity of the cells, but the latter were not capable of growing continuously in this solution.

6. The conclusion is drawn that, in accordance with the gradient theory previously advanced by the authors, the change in inductive capacity may be explained as a weakening of the mesodermalizing (M) principle in the cells, whilst the neuralizing (N) principle remains the same. A result of this change in the N/M balance would be that the spinocaudal action is weakened and the archencephalic effect is strengthened. At the same time the results show that the mesodermalizing action of the cells is dependent on external factors, whilst the neuralizing principle seems to be more stable. The results are compared with previous investigations on the changes in the inductive capacity of tissues caused by starvation, rapid growth, and malignancy.

## ACKNOWLEDGEMENTS

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## EXPLANATION OF PLATES

### PLATE 1

FIGS. 1–4. Inductive effect of HeLa cells grown in untreated ('active') serum: strong secondary tail structures, mostly regular in shape.

FIG. 5. Section of the larva shown in Fig. 1.

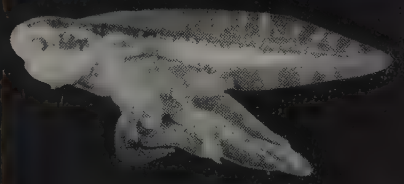
FIG. 6. Section of the larva shown in Fig. 2.

### PLATE 2

FIGS. 7–10. Example of the inductive effect of HeLa cells grown in heat-inactivated serum (30 minutes at 65° C.). In Figs. 9 and 10, a small fin is seen, otherwise the secondary structure consists of a firm protuberance, in which eye pigments are often discernible (Fig. 8).

FIG. 11. Section of the larva shown in Fig. 7.

FIG. 12. Section of the larva shown in Fig. 8.



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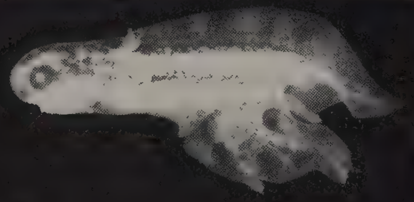

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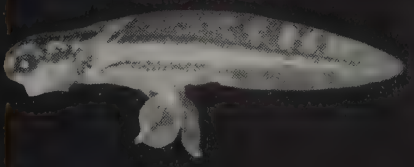

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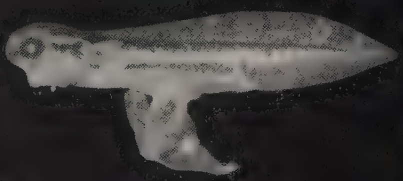

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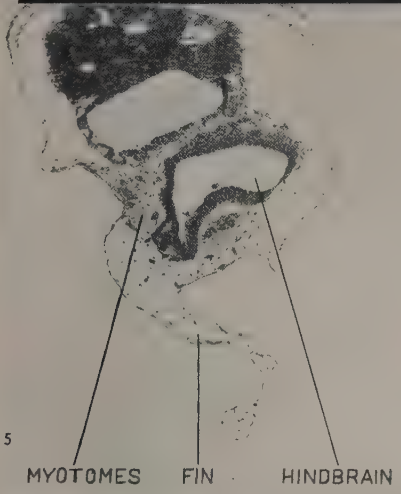

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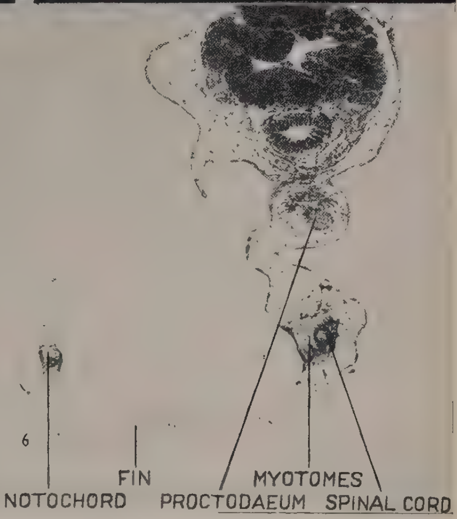

6

L. SAXÉN *and* S. TOIVONEN

*Plate 1*

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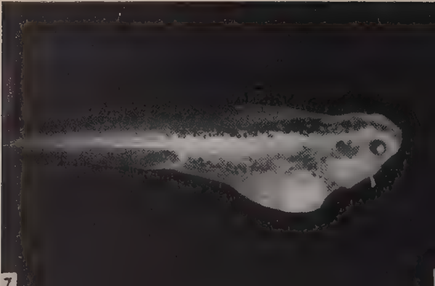

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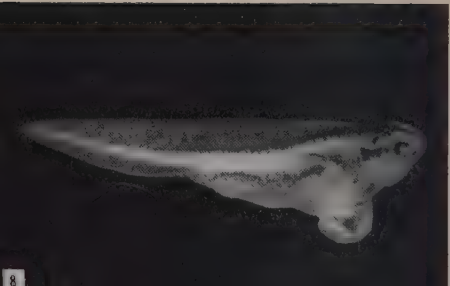

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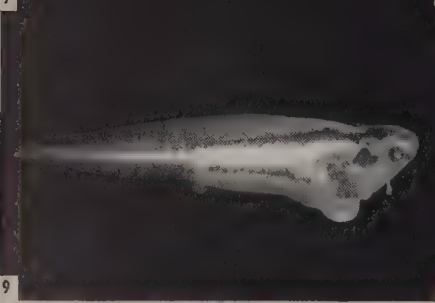

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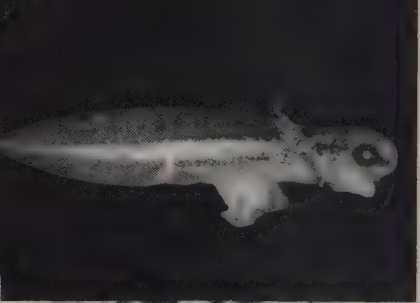

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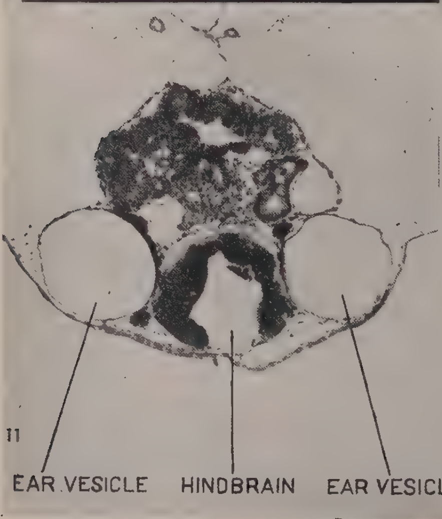

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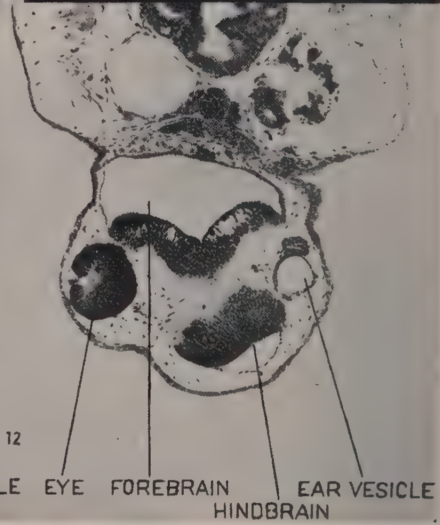
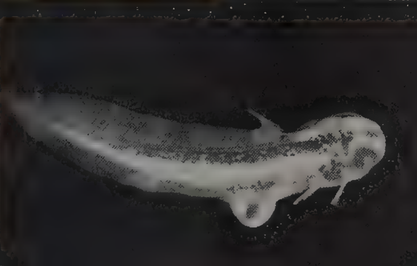


L. SAXÉN *and* S. TOIVONEN

*Plate 2*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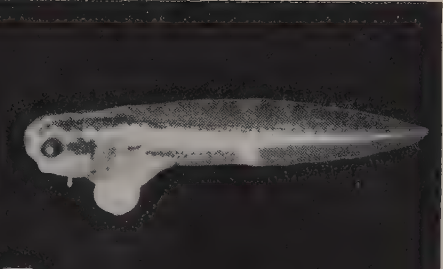
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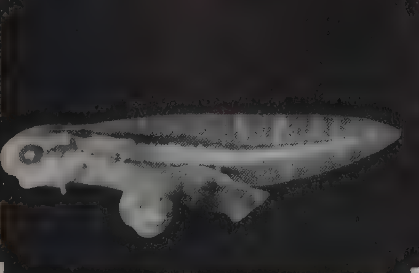
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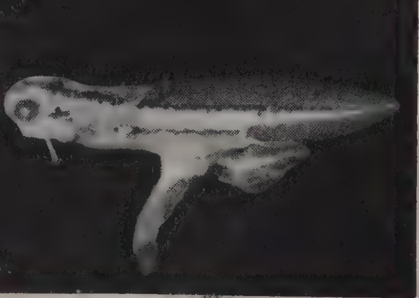
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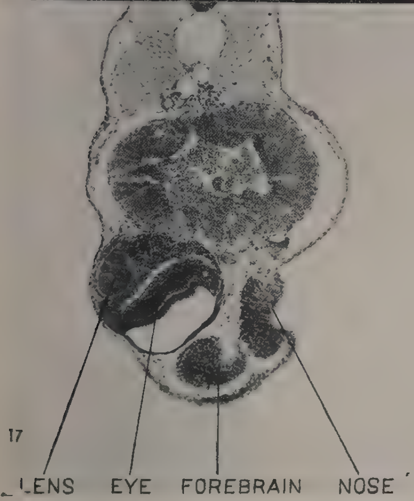
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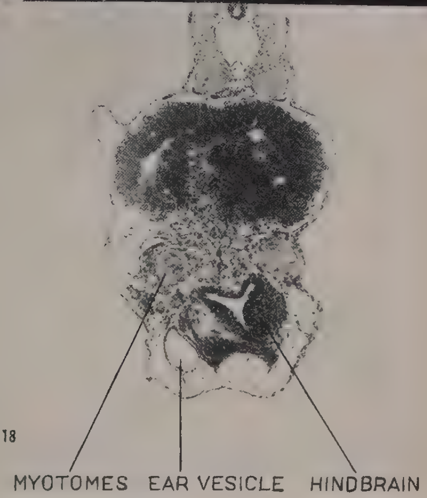
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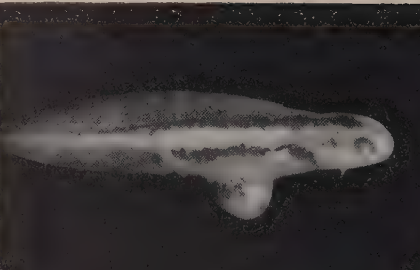
L. SAXÉN *and* S. TOIVONEN

*Plate 3*



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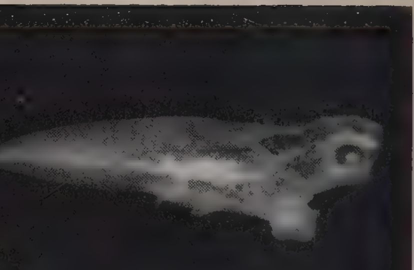

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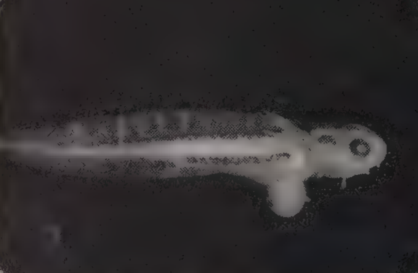

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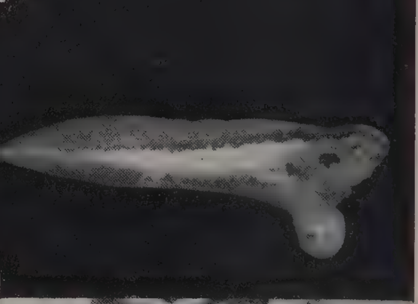

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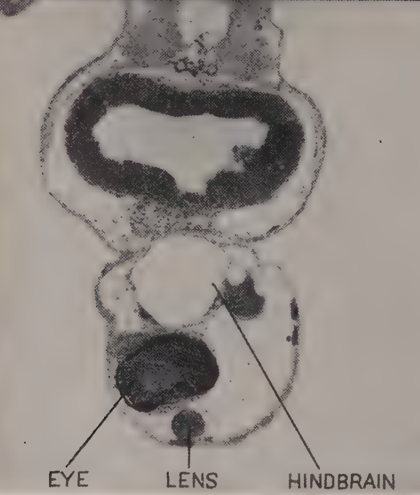

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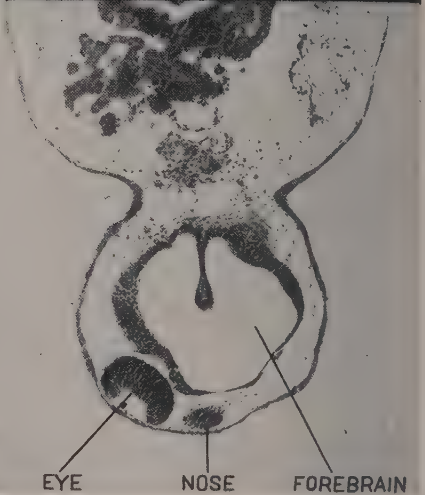

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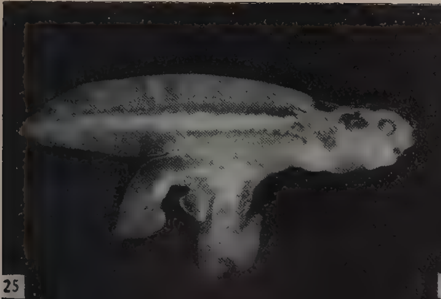
L. SAXÉN *and* S. TOIVONEN

*Plate 4*



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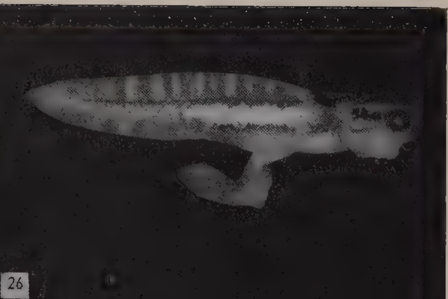

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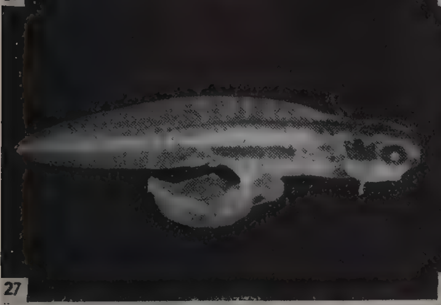

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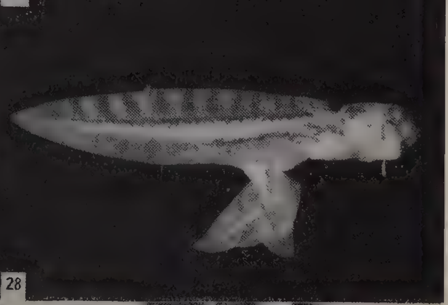

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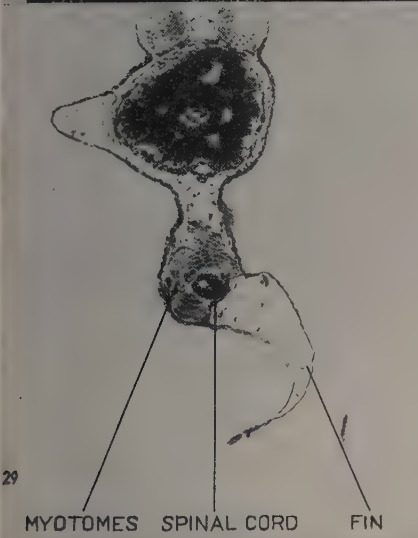

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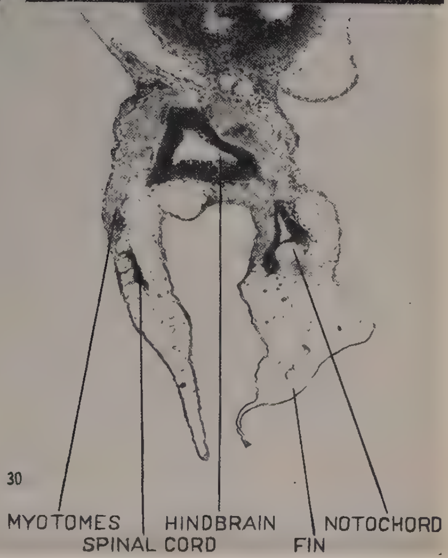

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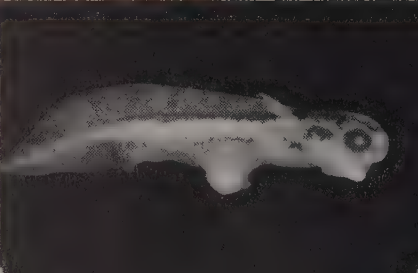
L. SAXÉN *and* S. TOIVONEN

*Plate 5*



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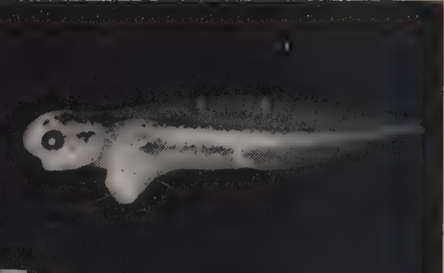

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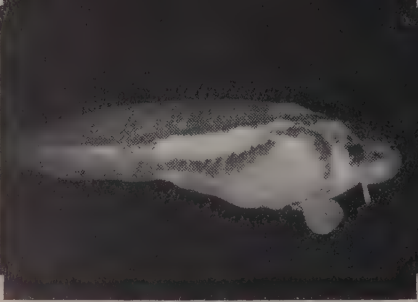

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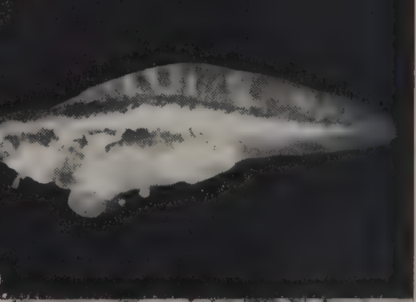

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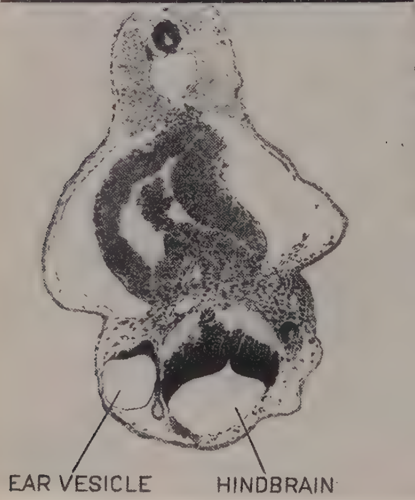

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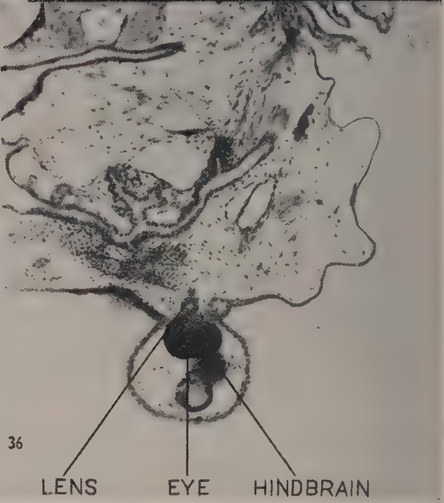

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L. SAXÉN *and* S. TOIVONEN

*Plate 6*

## PLATE 3

FIGS. 13–14. Inductive effect of HeLa cells grown in untreated ('active') serum after heating of the cells for 30 minutes at 65° C. No tail structures are observable.

FIGS. 15–16. Inductive effect of HeLa cells grown in untreated ('active') serum after heating of the cells for 30 minutes at 56° C. The secondary structures correspond to those observed in the control series (Plate 1).

FIG. 17. Section of the larva shown in Fig. 13.

FIG. 18. Section of the larva shown in Fig. 16.

## PLATE 4

FIGS. 19–22. Inductive effect of HeLa cells continuously grown in heat-inactivated serum (30 minutes at 56° C.). The secondary structures consist of firm protuberances, exhibiting eye pigments and often balancers (Fig. 20), but no tail structures.

FIG. 23. Section of the larva shown in Fig. 19.

FIG. 24. Section of the larva shown in Fig. 21.

## PLATE 5

FIGS. 25–28. Inductive effect of HeLa cells continuously grown in heat-inactivated serum (compare Plate 4) after being returned for 3 days to untreated ('active') serum. Conspicuous secondary tails correspond completely to those observed in the control series (Plate 1).

FIG. 29. Section of the larva shown in Fig. 27.

FIG. 30. Section of the larva shown in Fig. 28.

## PLATE 6

FIGS. 31–34. Inductive effect of HeLa cells grown for 4 days in protein-free growth medium (Parker's 199). The secondary structures are small, firm protuberances; no tail reactions are observable.

FIG. 35. Section of the larva shown in Fig. 32.

FIG. 36. Section of the larva shown in Fig. 33.

*(Manuscript received 16:v:58)*

# An Ultra-Violet Light Method for Producing Haploid Amphibian Embryos

*by* G. G. SELMAN[1]
*From the Institute of Animal Genetics, Edinburgh*

FOR several years in this laboratory, ultra-violet light has been used to produce haploid amphibian embryos, sometimes to provide haploid nuclei for transplantation experiments, as in Pantelouris & Jacob (1958). The ultra-violet method gives a high proportion of haploids, is very simple and speedy to carry out and involves the use of only inexpensive apparatus. Our method and results are described here to help others choose between this and alternative methods such as the use of toluidine blue by Briggs (1952), or X-rays by Lehman (1955) and Rugh (1939). Other methods are reviewed in Fankhauser (1937) and Drebinger (1951).

Eggs were taken from the oviducts of newt females and were placed close together in groups on cellophane squares to which they adhered by their jelly coats so that their animal surfaces were uppermost. The cellophane squares of unfertilized eggs were then placed in Petri dishes in air kept moist with a piece of damp filter paper at one side. The vas deferens were then excised from the male newt, broken into a shallow depression slide, and the sperm suspension diluted with approximately one equal volume of full strength Holtfreter's saline. All solid pigmented material and fat bodies, &c., were removed with needles or fine forceps under a binocular, to leave a uniform suspension of motile sperm in a drop about a millimetre deep and a centimetre across. This was placed in the uniform beam of ultra-violet light for the required time, usually about 2 minutes. The dose was given in stages of 30 seconds' duration between which the sperm suspension was stirred with a needle for a few seconds to reduce the numbers of sperm which would otherwise have been masked from the beam by sperm above them. Drops of treated sperm were then placed with a clean fine pipette on to the animal surface of the eggs, and after a further minute or two, one-tenth strength Holtfreter's saline was added. The following day the expanded jelly capsules of the embryos could easily be detached from the cellophane and from each other.

The ultra-violet source was a 125-watt MB/U-type high-pressure mercury vapour lamp manufactured by the General Electric Company Ltd., from which the outer glass envelope had first been cut away after carefully letting down the

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vacuum through a diamond scratch. The lamp was supported in a metal housing so that the quartz tube was 5 cm. to the side of a plane aluminium-surfaced mirror arranged so as to reflect light downwards through a fused quartz lens 13 cm. below the mirror to give a uniform area of ultra-violet illumination on the bench, which was 55 cm. below the lens at the lower intensity used. The spectral energy distribution curve for this lamp has a maximum round 2,600 Å. The approximate intensity of ultra-violet illumination incident upon the sperm suspension is quoted in the table of results for the so-called lethal range of the ultra-violet between wavelengths 2,100 Å and 3,200 Å. The figures were arrived at by calculation from the maker's spectral energy distribution curve for the source, together with a knowledge of the geometry of the apparatus. The same ultra-violet apparatus was also used by Edwards (1957) in this laboratory to induce gynogenesis in the mouse. Unfortunately the spectral energy-distribution curve for the lamp is shown incorrectly in that paper and does not correspond to the curve for the lamp as supplied by the manufacturer, because the energy contributed by the line spectrum has been ignored. However, the figures for the intensity of radiation, quoted in the same paper, appear to be correct.

## TABLE 1

| | *Ultra-violet dose* | | *Egg and embryo numbers* | | | | *Ploidy of individuals from chromosome counts* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Species* | *Intensity (ergs/cm.²/sec.)* | *Time (sec.)* | *Treated* | *Un-cleaved* | *Began to develop* | *Died in dev.* | $n \pm 2$ | $n+2n$ | $2n$ | *Others* | *No mitoses* |
| *T. palmatus* . | 20 | 35 | 11 | 6 | 4 | 1 | 3 | — | — | — | — |
| ,, | 20 | 45 | 6 | 4 | 2 | 0 | 1 | — | — | — | 1 |
| ,, | 20 | 60 | 10 | 7 | 3 | 0 | 3 | — | — | — | — |
| *T. vulgaris* . | 20 | 45 | 19 | 7 | 9 | 7 | 1 | — | — | — | 1 |
| ,, | 20 | 60 | 9 | 0 | 9 | 6 | 2 | — | — | — | 1 |
| ,, | 20 | 80 | 12 | 10 | 2 | 0 | 1 | — | — | — | 1 |
| ,, | 20 | 120 | 14 | 14 | 0 | 0 | — | — | — | — | — |
| *T. vulgaris* . | Control | 0 | 25 | 7 | 18 | 1 | — | — | 12 | — | 5 |
| *T. palmatus* . | Control | 0 | 20 | 4 | 16 | 0 | — | — | 14 | — | 2 |
| *T. palmatus* . | 8 | 60 | 5 | 1 | 3 | 0 | 2 | 1(10% $2n$) | — | — | — |
| *T. vulgaris* . | 8 | 60 | 5 | 0 | 1 | 0 | 1 | — | — | — | — |
| ,, | 8 | 120 | 6 | 5 | 1 | 0 | 1 | — | — | — | — |
| *T. alpestris* . | 8 | 30 | 31 | 11 | 20 | 13 | — | — | 6 | 1(20) | — |
| ,, | 8 | 60 | 190 | 21 | 169 | 104 | 20 | 2 | 18 | $3\left\{\begin{matrix}3n\\2n+4n\\18\end{matrix}\right\}$ | 3 |
| ,, | 8 | 90 | 44 | 17 | 27 | 11 | 12 | — | 1 | 1(18) | 2 |
| ,, | 8 | 90 or 150 | 41 | 6 | 35 | 9 | 9 | — | 12 | — | 5 |
| ,, | 8 | 120 | 52 | 10 | 42 | 28 | 8 | — | 5 | — | — |
| ,, | 8 | 180 | 29 | 5 | 24 | 7 | 9 | — | 4 | $2\begin{pmatrix}14,\ 15\\22\end{pmatrix}$ | 2 |

The numbers in that part of the table which shows the results of the chromosome counts, indicate numbers of embryos throughout, except that the figures in parenthesis indicate chromosome numbers found at metaphase in individual embryos.

There is no reason why other ultra-violet sources may not be used for producing haploids, provided that the source is contained in a quartz envelope, all glass lenses or filters are excluded, sufficient energy is radiated near 2,600 Å, and that an appropriate dose is given to the sperm. The appropriate dose can quickly be found from observations made under a low-power microscope on the decrease of motility of a healthy sperm suspension after ultra-violet radiation. The dose to induce subsequent gynogenesis is about half that required to render nearly all the sperm immotile. This dose will itself produce a noticeable decrease of motility.

The table shows the numbers of haploids produced in experiments with three species of newt activated with their own treated sperm. Haploids are recorded in this table only when 12 ± 2 metaphase chromosomes were counted in stained and fixed preparations. Haploids can also be recognized by their smaller nuclei and melanophores (see Fankhauser, 1945). Permanent cytological preparations were made by the tail-tip method as in Fankhauser (1945) with Meyer's acid haemalum, or for earlier stages the aceto-orcein method of Lehman (1955) was used. Temporary squashes by Lehman's method are particularly useful for finding the ploidy of a piece of tissue in about 10 minutes before similar tissues from the same embryo are used in transplantation experiments. Preparations may subsequently be made permanent. The tail-tip method was used almost exclusively for the experiments with *Triturus palmatus* and *T. vulgaris*.

The majority of haploid embryos and larvae had a uniform 12 chromosomes in each metaphase counted. A few others had individual metaphases with definitely 11 or 13 chromosomes in addition to those with 12. In the larvae of *T. palmatus* and *T. vulgaris* which developed from treated sperm only haploid nuclei were found, except that one larva also had about 10 per cent. diploid cells. With *T. alpestris* there was less success, but with sperm dosed from $1\frac{1}{2}$ to 3 minutes at 8 ergs/cm.$^2$/sec., considerably more than half the embryos which developed were haploids. It might be possible to improve this percentage of haploidy by washing, centrifuging and resuspending the sperm before treatment and by working out the optimum sperm concentration to irradiate, but the present method was speedy and gave a sufficiently high proportion of haploids for our purposes.

With less success, an attempt was made to produce haploids of *T. palmatus* by treating the animal surface of the unfertilized eggs with doses of ultra-violet light, which were about 10 times greater than the doses used for treating sperm, and subsequently fertilizing the eggs artificially with untreated sperm. From 76 eggs so treated 26 started to develop, which was about half the proportion which developed from the untreated controls. Among these 26, 2 were haploid, 15 were diploid, one was triploid, one was a diploid-triploid mosaic, and another was a multiform aneuploid with chromosome numbers varying between 14 and about 36. It is likely that the cytoplasm of the egg above the female pronucleus absorbs ultra-violet light strongly and so protects the female pronucleus. More penetrating ionizing radiations (e.g. X-rays or $\gamma$ rays) are doubtless

more suitable than ultra-violet light for inducing androgenesis. My thanks are due to Dr. M. Fischberg for his initial suggestion to develop the ultra-violet method, and for his instruction on certain techniques of amphibian embryology.

## SUMMARY

Details are given of a method for producing haploid newt embryos by activating eggs with ultra-violet-light-treated sperm. A high proportion of haploids was obtained with three species of *Triturus*.

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# Skeletal Regeneration in Amphibians

*by* R. J. GOSS[1]

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WITH ONE PLATE

## INTRODUCTION

THE failure of *de novo* regeneration of extirpated bones in adult urodeles has been described repeatedly. Wendelstadt (1901) reported the absence of skeletal regeneration following the removal of either the ulna or radius from the otherwise intact limbs of salamanders. Reed (1903) and Morrill (1918) likewise failed to obtain regeneration of previously excised fibulae in salamander hind limbs. Similar negative results were reported by Schaxel & Böhmel (1928) and Böhmel (1929) following the removal of femurs from axolotls. Gebauer (1933) recorded no regeneration of extirpated bones in *Triton cristatus*, and Goss (1956) and Rieck & Rudich (1956) noted that ulnae were not replaced after their removal from the limbs of adult *Triturus viridescens*. In contrast to the situation in adults, Swett & Parsons (1929) have reported the regeneration of completely removed humeri and shoulder girdles in the young larvae of *Amblystoma punctatum*.

In the present research, the problem of skeletal regeneration has been reinvestigated, both extensively and intensively. Firstly, the responses of larval urodeles and anurans to skeletal extirpation has been compared with those of adult urodeles. Secondly, on the theory that the absence of bone regeneration in adults might be due to the more highly differentiated condition of their tissues, as illustrated, for example, by the presence of ossified rather than cartilaginous parts, the replacement of skeletal elements removed from adult limb regenerates of various ages has been analysed. Finally, the possibility that nerves might influence skeletal restitution, as they do limb regeneration, has been investigated.

## MATERIAL AND METHODS

In order to study comparatively the replacement of excised skeletal parts, tibiae were removed from the hind legs of *Rana pipiens* tadpoles, and ulnae from the forelimbs of larval *Amblystoma maculatum* and adult *T. viridescens*. All operations were performed on animals anesthetized with MS 222 (Sandoz). Bones were removed intact through longitudinal incisions in the skin of the limbs, and the wounds were subsequently sutured whenever possible.

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Other operations were similarly performed on forelimb regenerates of adult *T. viridescens.* Limbs were originally amputated through the proximal humerus and allowed to regenerate. The cartilaginous ulnae were then extirpated from these limb regenerates after 6, 7, 8, 10, 12, 14, and 16 weeks. The operated limbs of each group of animals were prepared for examination 8 weeks later.

The possible role of nerves was investigated wherever skeletal regeneration was normally observed to occur, namely, in the limbs of larval *Amblystoma* and in the regenerated extremities of adult *Triturus.* Ulnae were removed and the limbs simultaneously denervated at the shoulder. In order to preserve the nerveless condition, denervations were repeated midway through the experiment (10 days in larval *Amblystoma,* 3 weeks in adult *Triturus*). In these cases, the contralateral limbs, which remained fully innervated, served as controls.

All limbs were prepared for examination by staining *in toto* in methylene blue following fixation in 70 per cent. alcohol (95 c.c.) and formalin (5 c.c.). This method selectively stains cartilaginous elements, the visualization of which was facilitated by dissecting away the skin and musculature. The extent of skeletal replacement was determined by measuring the lengths of the regenerated elements as fractions of the original lengths. The figures thus obtained in a given experimental series were then averaged to yield an overall percentage skeletal regeneration.

## RESULTS

### *Skeletal regeneration in larvae* v. *adults*

The ability of anuran larvae to regenerate missing skeletal parts was ascertained in *R. pipiens* tadpoles. Tibiae were removed from 43 left hind legs which varied in degree of development from small (3 mm.), immobile limbs exhibiting only incipient digit formation, to large (16 mm.), mature legs on tadpoles nearing metamorphosis. To determine if skeletal regeneration is related to the ability of limbs to regenerate entire extremities, the opposite right legs were simultaneously amputated midway between the knee and ankle. Of the latter, a total of 36 regenerated legs, and 7 of the larger ones failed to do so. With reference to the left legs, only 6 had regenerated the missing tibiae at the end of 4 weeks, and of these, 5 had replaced only the distal halves of the tibiae while one possessed a complete skeletal regnerate. Although all 6 of these limbs were from animals which had given rise to complete regenerates after amputation of their right legs, the large number of other cases which had also regenerated right legs but had not replaced their left tibiae precludes any definite correlation between the two processes. Apparently the capacity to regenerate extirpated skeletal elements is lost earlier in the development of the leg than is the ability to regenerate a complete limb.

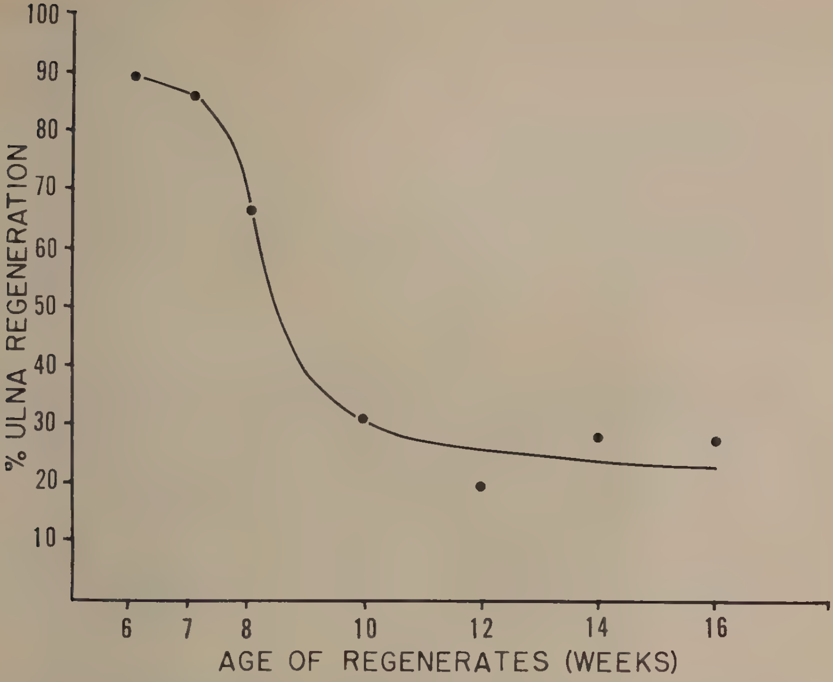
In *A. maculatum,* ulnae were removed from the right forelimbs of 17 larvae 2·5–3·5 cm. long. Three weeks later, 7 limbs had failed to replace the missing ulnae. However, in the remaining cases, one limb had formed a cartilaginous element about three-quarters normal length, and the other 9 had completely regenerated new ulnae (Plate, fig. A). Thus, there was 57 per cent. ulna regeneration in this experimental series. In all of the successful cases, in addition to the formation of new ulnae, small supernumerary digits were also produced laterally.

Experiments on adult urodeles involved the extirpation of ulnae from the forelimbs of *T. viridescens*. In all 36 cases no skeletal regeneration occurred. In view of the total absence of regeneration under these conditions, two attempts were made to promote bone replacement in the adult newt. In one experiment, the ulnae in 15 limbs were first fractured in 3 or 4 places along their lengths. Six weeks later, when cartilaginous calluses had formed, the ulnae were removed. After another 8 weeks, examination of the limbs revealed no signs of chondrification, indicating that the simple presence of cartilage instead of bone does not suffice to promote skeletal regeneration in adult urodeles. In another experiment, 3-week-old blastemas from amputated forelimbs of adult *Triturus* were minced and transplanted homoplastically to 32 limbs from which the ulnae had been removed. Despite the presence of undifferentiated cells, no chondrification occurred during the 8-week duration of the experiment. These negative results are consistent with those obtained by Gebauer (1933) following similar experiments.

### *Ulna regeneration in adult forelimb regenerates*

Inasmuch as developing limb regenerates of adult urodeles resemble larval limbs, there is reason to expect that skeletal elements might be replaced following their removal from immature regenerates, and that as the latter gradually acquire adult characteristics their ability to regenerate skeletal parts might diminish. Accordingly, this hypothesis was tested by removing the ulnae from groups of limb regenerates varying in age from 6 to 16 weeks, and the extent of skeletal replacement was assessed 8 weeks later. As graphically illustrated in Text-fig. 1, young regenerates exhibited considerable ability to replace missing ulnae, while progressively older ones gradually lost this capacity.

Following the extirpation of ulnae from 6-week-old limb regenerates, there was 89 per cent. skeletal regeneration in 40 cases. Similar experiments on 7-week-old regenerates yielded 86 per cent. ulna replacement in 28 cases (Plate, fig. C). In the 8-, 10-, and 12-week-old regenerates (16 limbs each), the extent of ulna regeneration was respectively 66 per cent., 31 per cent., and 19·7 per cent. (Plate, fig. D). The 14-week-old regenerates (14 cases) and 16-week-old regenerates (11 cases) each resulted in about 28 per cent. ulna replacement. With reference to those limbs possessing completely regenerated ulnae, the numbers in each group of animals gradually decreased from 34 out of 40 (85 per cent.) in the 6-week-old regenerates to one out of 11 (9 per cent.) in the 16-week-old regenerates.



TEXT-FIG. 1. Percent skeletal regeneration after removal of ulnae from adult *Triturus* forelimb regenerates of various ages.

## *The rôle of nerves in skeletal regeneration*

As described above, ulna regeneration takes place in the forelimbs of larval *Amblystoma* and in the regenerated arms of adult *Triturus*. In order to ascertain if this process is dependent upon influences emanating from nerve fibres, limbs of the above types were denervated simultaneously with the ablation of their ulnae.

In 17 *Amblystoma* larvae, the left arms were denervated at the beginning of the experiment and also 10 days later, while the right arms served as controls. In 7 of the nerveless limbs, from which the ulnae had been removed, extensive regression occurred, precluding analysis of the results. In view of the experiments of Thornton & Kraemer (1951), resulting in the regression of denervated limbs of *Amblystoma* larvae following injury to the internal tissues, the above occurrence was not unexpected. In 10 remaining limbs, however, regression was either absent or limited, and in these circumstances no ulna regeneration had occurred after 3 weeks (Plate, fig. B). The control limbs, as previously described, manifested 57 per cent. ulna reconstitution in the same length of time. It is concluded, therefore, that nerves are necessary for skeletal regeneration in larval urodeles.

Similar experiments on adult *Triturus* included 40 limb regenerates 6 or 7 weeks old. Denervations were performed at the time of ulna extirpation, and also 3 weeks later. The limbs were fixed for examination after 6 weeks. Regression of the regenerated limbs, as in the previous experiment, occurred in 27 cases. Of the remaining limbs, 9 showed no indications of ulna replacement, while in three instances partial regeneration had occurred (Plate, fig. E), and in one case total replacment of the ulna was observed. In view of the normally high incidence of ulna regeneration in limb regenerates of these ages (Text-fig. 1), it is probable that the few exceptional cases can be accounted for by incomplete denervation of the limbs. It seems evident, therefore, that nerves are essential for the regeneration of skeletal parts in adult limb regenerates.

## DISCUSSION

In the present investigation it has been shown that skeletal regeneration does not occur in adult urodeles, and is rare in all but the youngest larval anuran legs. Larval urodeles and the young limb regenerates of adult urodeles, however, are capable of replacing excised skeletal elements. In attempting to explain this pattern of regenerative ability, several possibilities suggest themselves.

It is obvious that skeletal regeneration occurred only when the previously removed structure was cartilaginous. In *Amblystoma* larvae, the skeletal elements were composed entirely of cartilage, and in the young limb regenerates of adult *Triturus* the extirpated ulnae were cartilaginous. Indeed, as such regenerates became older, the gradual decrease in the percentage of ulna regeneration was in general correlated with a progressive increase in calcification of the skeletal parts. However, in view of the usual failure of the cartilaginous tibiae of anuran tadpoles to be replaced, and the absence of ulna regeneration following the extirpation of adult *Triturus* ulnae which had formed chondrified calluses as a result of previous fractures, the hypothesis that skeletal regeneration is solely dependent upon the prior existence of cartilage becomes untenable.

A more plausible hypothesis relates to the general state of differentiation of the tissues involved in skeletal regeneration. This, in turn, may be correlated with the fact that skeletal replacement takes place only in limbs which are capable of regeneration and also possess cartilaginous skeletal elements. Thus, the extremities of *Amblystoma* larvae, and the young regenerated limbs of adult *Triturus* fulfil both of these criteria. However, the tadpole hind limb seems to be an exception inasmuch as its ability to regenerate after amputation outlasts its capacity to replace missing skeletal parts. Nevertheless, it is possible, as suggested by Swett (1929), that skeletal regeneration is similar in many respects to total limb regeneration.

This view is further substantiated by the observation that in *Amblystoma* larvae, ulna replacement was attended in most instances by the regeneration of accessory appendages. Moreover, skeletal regeneration in adult amphibians, proximal to the level of limb amputation, has been observed occasionally in association with the regeneration of limbs lacking some or all of their bones

(Morrill, 1918; Bischler, 1925; Brunst, 1932; Goss, 1953, 1956). In these cases, the abundance of undifferentiated blastema cells presumably facilitated bone regeneration. The parallel effects of nerves in both phenomena also emphasizes their similarities. As in the case of total limb regeneration, nerves are essential for the *de novo* reconstitution of ulnae in the forelimbs of *Amblystoma* larvae and in the regenerated arms of adult *Triturus*.

In conclusion, it is postulated that skeletal regeneration necessitates the presence of a sufficient number of cells capable of undergoing chondrogenesis. Following the extirpation of a skeletal element, these cells accumulate in the space previously occupied by the missing structure and form there a blastematous mass which subsequently undergoes chondrification and morphogenesis. Obviously, the distribution of these potential chondroblasts must be sufficiently dispersed to ensure the persistence of enough residual cells from which a new skeletal structure can develop. Presumably, in the larval *Amblystoma* limb and in the young regenerates of adult *Triturus* limbs, such a distribution of cells exists. In the normal adult *Triturus* limb, chondrogenic cells are evidently restricted to the periosteum, where, for example, they are capable of promoting the formation of a cartilaginous callus in the event of fracture. However, the extirpation of such bones removes virtually all of these cells, thus precluding skeletal regeneration through the absence of source material.

## SUMMARY

1. In order to investigate the replacement of skeletal elements in the Amphibia, bones were removed from the extremities of anuran tadpoles, and larval and adult urodeles. Following the extirpation of tibiae from the hind limbs of *R. pipiens* tadpoles, skeletal regeneration occurred in only a few of the smaller legs. Ulnae removed from the forelimbs of adult *T. viridescens* were not replaced even when the bone had been previously fractured or when it was replaced by blastema tissue from amputated arms. Cartilaginous skeletal regeneration did occur, however, after extirpation of ulnae from the forelimbs of larval *A. maculatum*. Likewise, ulnae removed from young forelimb regenerates of adult *Triturus* were regularly reconstituted, but as such regenerates grew older the percentage of ulna replacement progressively diminished.

2. In view of these results, it is concluded that in order for skeletal regeneration to occur the extirpated structure must be cartilaginous, and it must be removed from a limb otherwise capable of regeneration after amputation. This, together with the evidence that skeletal regeneration, like total limb regeneration, is dependent upon adequate innervation, substantiates the contention that these two phenomena are different manifestations of the same process. It is suggested that the absence of skeletal regeneration is dependent upon the developmental state of surrounding tissues, such that in larval or recently regenerated urodele limbs sufficient potential chondroblasts persist after skeletal extirpation

to ensure replacement, while in adult limbs chondrogenic cells, being restricted to the periosteum, are nearly all removed with the bone.

## ACKNOWLEDGEMENTS

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## EXPLANATION OF PLATE

All photographs are of whole mounts of limbs in which the cartilage has been selectively stained with methylene blue. Magnification: ×15. *Abbreviations*: H, humerus; R, radius; U, ulna.

FIG. A. Right arm of larval *Amblystoma* from which the ulna had been extirpated 3 weeks previously. A new ulna (U) has been formed *in situ*, and a supernumerary digit (arrow) has regenerated.

FIG. B. Left arm of same animal which was denervated when the ulna was removed. After 3 weeks no regeneration of the ulna has occurred.

FIG. C. Adult *Triturus* forelimb regenerate following ablation of its ulna at 7 weeks of age. After another 8 weeks, a new ulna (U) has been regenerated.

FIG. D. Adult *Triturus* forelimb regenerate from which the ulna was removed 12 weeks after amputation. Eight weeks later no skeletal regeneration has taken place.

FIG. E. Adult *Triturus* forelimb regenerate which at 6 weeks of age was deprived of its ulna and denervated. After another 6 weeks only a very small skeletal element (U) has regenerated.

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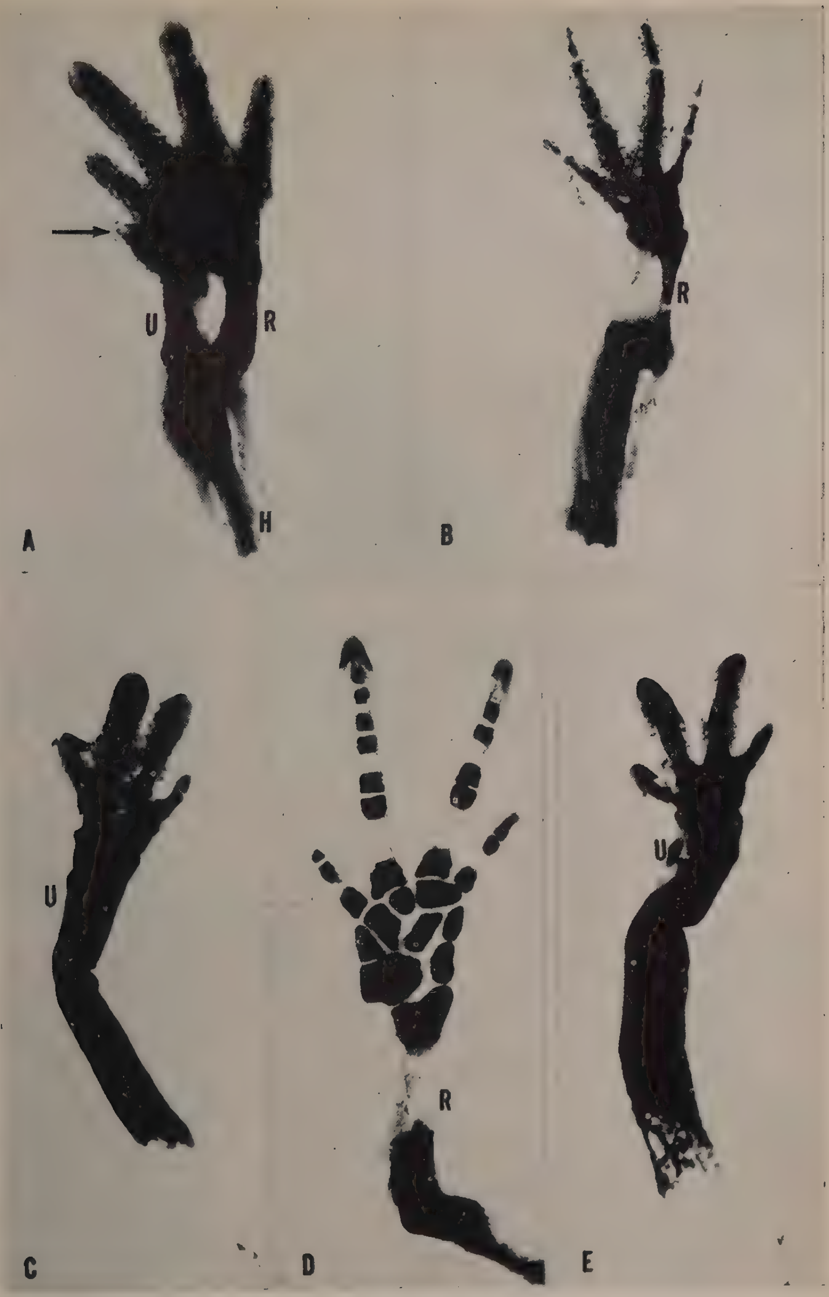

R. J. GOSS

# Factors Affecting Vertebral Variation in Mice

## 4. Experimental Proof of the Uterine Basis of a Maternal Effect

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*From the Royal Veterinary College, University of London*

WITH ONE PLATE

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### INTRODUCTION

THE historic controversy between 'spermists' and 'ovists' ended towards the close of the eighteenth century in a partial victory for both sides: sperm and egg were shown to be alike necessary for the genesis of a new individual. But the question of the *relative* biological contribution of the male and female parent remained open.

A century later the biometrical school of Karl Pearson showed that in human stature, and a number of related characters, the respective influences of the mother and the father upon the offspring were of equal strength. The rediscovery at this time of Mendel's work seemed to offer the final and decisive proof of sex equality in heredity, for according to Mendel's system the number of hereditary factors supplied by each parent was of necessity equal.

*Sex linkage*

Somewhat paradoxically, Morgan's discovery in 1911 of the chromosomes as the bearers of the Mendelian factors was based on the first known exception to the rule of equality, namely, on sex linkage. In this case the female specifically influences her sons through the X-chromosome which she transmits to them, so that sex linkage may be described as a kind of maternal effect, mediated through the sex chromosomes and manifest in the male progeny only.

It is customary, however, to reserve the term 'maternal effect' for other, and more lately discovered, exceptions to the rule of sex equality, where the route of transmission is non-chromosomal and the specific influence of the maternal parent is detectable in the female, no less than in the male, progeny. In Table 1 is set out a classificatory scheme of the various forms of maternal effect found in mammals, in which sex-linkage is included.

If reciprocal crosses between two contrasting types yield $F_1$ progenies which

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differ from one another, the difference is almost certainly due to a maternal effect, although the possibility of a paternal effect (exerted through the Y-chromosome or through the sperm cytoplasm) should not be forgotten. Reciprocal differences due to maternal effects are usually in the direction of the mother's type (matroclinous), more rarely in the direction of the father's type (patroclinous).

TABLE 1

*Classification of maternal effects in mammals*

| *Category of maternal effect* | | *Stage at which effect is exerted* | *Route by which effect is exerted* | *Sex of progeny on which effect is exerted* |
|---|---|---|---|---|
| Germinal | Chromosomal | Before fertilization | X-chromosome | Male |
| | Cytoplasmic | Before fertilization | Cytoplasm<br>Other non-chromosomal cellular elements | Male and female |
| Somatic | Uterine | After fertilization, before birth | Oviduct<br>Uterus | Male and female |
| | Milk | After birth | Colostrum<br>Milk | Male and female |

### *'Germinal' and 'somatic' maternal effects*

Routes of hereditary transmission which lie through the germ-cells are not confined to the chromosomes. Non-chromosomal germinal transmission is termed 'cytoplasmic', on the reasonable presumption that the factors responsible for it are located in the cytoplasm of the egg rather than, say, in the nuclear sap or in the cell membrane. The other category of maternal effect may be termed 'somatic', and comprises those routes which by-pass the germ-cells. In mammals this category includes effects exerted on the unborn young through the uterine environment, and effects exerted post-natally through the milk. A widely known example of the latter mode of transmission is the 'milk factor' which confers susceptibility to spontaneous mammary cancer in some strains of mice.

### *Uterine effects*

The only maternal effects known definitely to act through the uterine environment are effects upon foetal growth, as demonstrated by Venge (1950) in rabbits and by Hunter, Adams, & Rowson (1954) and Hunter (1956) in sheep. We can suppose these effects on growth to be either mechanical or, in the simplest sense, nutritional. Restriction either of intra-uterine space or of the quantity of nutriment available to the foetus could be sufficient to set a limit upon growth. Evidence has so far been lacking that a specific morphogenetic modification, concerning a detail of form rather than the promotion or inhibition of overall growth, can be brought about by the mother through the uterine environment. In this paper we describe the results of a successful search for such evidence (see

McLaren & Michie, 1958, for a preliminary report of the work). A maternal effect upon the number of lumbar vertebrae in mice has been analysed by the method of egg-transfer and found to be uterine.

### *The vertebral column of the mouse*

In the axial skeleton of the mouse 26 pre-sacral vertebrae are commonly found. This number is subject to variation, as also is its typical division into 7 cervical, 13 thoracic, and 6 lumbar vertebrae. In the material used in the present work variation at the cervico-thoracic and thoraco-lumbar borders (i.e. the atypical presence or absence of ribs) is negligible, so that the number of lumbar vertebrae can be taken as a direct indication of whether or not sacralization of the 26th vertebra has occurred. When vertebra 26 is sacralized, 5 lumbar vertebrae are observed as illustrated in the Plate. When it is not, the number of lumbar vertebrae is 6. Sacralization can occur on one side only, but the frequency of asymmetrical animals is less than would be expected if the two sides were independent.

A sex difference is consistently encountered. Sacralization of vertebra 26 is commoner in male than in female mice. Sacralization is also affected by some factor of the mother's physiology which fluctuates with time, resulting in a correlation between litter-mates (McLaren & Michie, 1954).

### *Previous work*

In some inbred strains of mice the majority of animals have 6 lumbar vertebrae; in others the contrasting type, with 5 lumbar vertebrae, predominates. In reciprocal crosses between the C57BL strain (6 lumbar vertebrae) and a substrain of the C3H strain showing a high frequency of animals with 5 lumbar vertebrae, Russell & Green (1943) and Green & Russell (1951) found that the $F_1$ hybrid young tended to resemble the maternal strain. The difference was apparent in the female progeny and so could not be attributed to sex linkage. Since the character is determined before birth, the effect cannot be exerted through lactation, and must therefore, if maternal, be either cytoplasmic or uterine.

In an attempt to determine more precisely the route by which the mother might influence the number of lumbar vertebrae of her young, Russell (1948) transplanted ovaries from females of the 129 strain into $F_1$ hybrid hosts (129 × BALB/c) which were then mated to 129 strain males. In strain 129 the three vertebral types (5, asymmetrical, 6) occur in roughly equal proportions. The 129 strain progeny raised in hybrid foster mothers proved to have a lower average number of lumbar vertebrae than 129 strain progeny raised in 129 mothers. This experiment shows an effect of the host upon vertebral type; but since with ovarian transplantation the host provides not only the uterus in which the young are gestated, but also the materials from which the cytoplasm of the eggs is elaborated, the result is compatible with either a uterine or a cytoplasmic effect. It does, however, narrow the field of interpretation by showing that the effect is *either* uterine *or* due to a cytoplasmic factor which can be acquired by the oocyte through somatic contact of the ovary with a host animal.

Using the same combination of strains, Runner (1953, 1954) transferred eggs of the 129 strain into BALB/c foster mothers. The 26th vertebra is not sacralized in mice of the BALB/c strain. Eighteen mice from transferred eggs were raised, and it appears that they had a *higher* average number of lumbar vertebrae than control 129 strain mice, in contrast with Russell's findings using ovarian transplantation into hybrid hosts. But the statistical significance which is claimed for this comparison is vitiated by the assumption made that the sacralization of the two sides of the 26th vertebra can be counted as two independent events. Allowance for the correlation between sides reduces the apparent significance of the result to below the conventional level. It would presumably be yet further reduced if allowance were also made for correlation between litter-mates. The litter relationships of the animals were not stated.

Green & Green (1953) attempted by egg transfer to analyse a difference in vertebral type between reciprocal hybrids of the DBA/1 (mainly 5 lumbar vertebrae) and C57BL (mainly 6 lumbar vertebrae) inbred strains. The $F_1$ hybrid progeny showed intermediate percentages, but tended to resemble the *paternal* strain. It is, of course, possible here that the patroclinous effect is exerted by the *maternal* parent, but in a direction opposite to her own type. In order to test the possibility that such an effect was involved, and that it was exerted through the uterine rather than the cytoplasmic route, Green & Green arranged by means of unfertilized egg transfer that the two kinds of hybrid embryos should develop in foster mothers of the BALB/c strain. The reciprocal difference in control $F_1$ hybrids was small, and on the numbers of transferred mice which were raised there is no significant difference between reciprocals after transfer. Nor, however, is there any significant effect of transfer upon vertebral type, although in one of the two types of hybrid there is a small, subsignificant effect in the uterine direction. We are indebted to Dr. E. L. Green for sending us his numerical data.

In the light of the investigations summarized above, it seemed to us that the question concerning the cytoplasmic or uterine nature of maternal effects upon vertebral type remained open. We therefore undertook the experiment which is described below.

## MATERIALS, METHODS, AND TERMINOLOGY

In the earlier papers of this series (McLaren & Michie, 1954, 1955, 1956*a*) we have described work which was primarily undertaken in order to locate material suitable for the present experiment, and to disclose ancillary causes of vertebral variation which might have to be taken into account in its design and evaluation. On this basis the C57BL/How (mainly 6 lumbar vertebrae) and C3H/Bi (mainly 5 lumbar vertebrae) substrains were chosen to represent the C57BL and C3H inbred strains. A series of reciprocal crosses confirmed the existence of a matroclinous effect. As ancillary causes of variation, sex and substrain differences were implicated, the latter being due to secular drift in the underlying genetic properties of substrains. In addition a tendency was found for anomalous individuals to be 'clumped' into the same litters, resulting in a corresponding heterogeneity between different litters of the same mating. The statistical consequence of this litter-mate correlation is that litters rather than individual mice must be taken as the units of analysis. Its biological explanation presumably lies in some element of the mother's physiology which fluctuates during her lifetime. This fluctuation was, however, found not to be systematically related to maternal age, to birth order, or to litter size.

The technique used for transferring fertilized eggs has been described elsewhere (McLaren & Michie, 1956*b*). Eggs were taken from donors 3 days after the finding of copulation plugs and transferred to multiparous recipient females which had shown copulation plugs 2 days previously. Most of the experimental and control mice were classified for vertebral type by X-ray photography at a variable time after weaning. In a few cases of animals which died in the intervening period, vertebral type was determined by means of an alizarin transparency. The two methods of classification had previously been shown to give identical results (McLaren & Michie, 1954).

Vertebral type is described by the same set of symbols as previously used, namely:

5/5 = five lumbar vertebrae on each side.
6/5 = six lumbar vertebrae on the left side and five on the right.
5/6 = five lumbar vertebrae on the left side and six on the right.
6/6 = six lumbar vertebrae on each side.

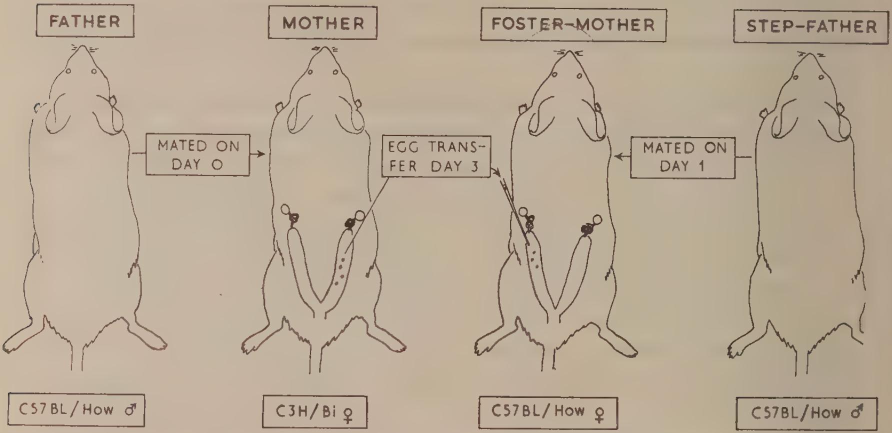
## THE PLAN OF THE EXPERIMENT

As stated above, preliminary crosses had confirmed that the majority of the $F_1$ hybrids of the C3H/Bi ♀ × C57BL/How ♂ cross resembled C3H/Bi mice in having 5 lumbar vertebrae, while the majority of the $F_1$ hybrids from the reciprocal cross resembled C57BL/How mice in having 6 lumbar vertebrae.

The main experiment to be described consisted of the transfer (see Text-fig. 1) of $F_1$ hybrid eggs obtained from C3H/Bi ♀ × C57BL/How ♂ matings into uterine foster mothers of the *paternal*, i.e. C57BL/How, substrain. If the matroclinous effect is cytoplasmic we should expect the transfer procedure to be without effect upon the offspring so obtained, which should therefore resemble the offspring of control C3H/Bi ♀ × C57BL/How ♂ matings. If, however, the effect is uterine we expect the offspring obtained from the transfers to resemble control offspring of the reciprocal cross, C57BL/How ♀ × C3H/Bi ♂, and to consist, for the most part, of 6/6 mice.

The recipient C57BL/How females had been mated to males of their own strain. $F_1$ hybrid young derived from transferred eggs could be distinguished by their agouti coat colour from their non-agouti litter-mates of native origin.

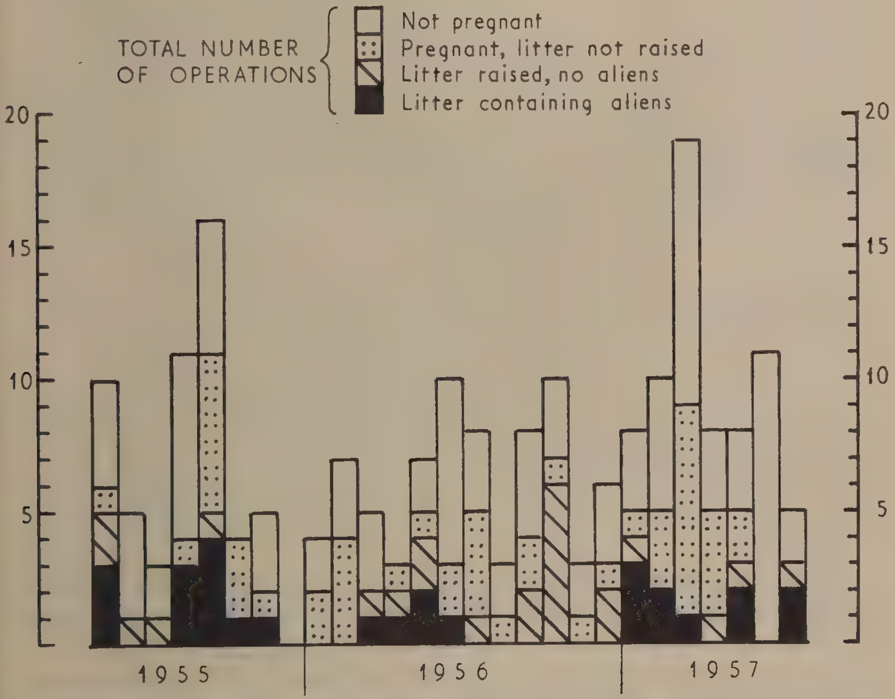
Since even with recipients of high fertility and fecundity the yield of live young from eggs transferred by our technique was expected to be only in the region of 20–25 per cent. (see McLaren & Michie, 1956*b*), and since in addition the substrains to be used were known to be of very low fertility and fecundity, we anticipated that the experimental data would accumulate slowly over a long period of time. In order to control the possible effects upon vertebral type of genetic drift and of environmental changes (for example seasonal) during the experimental period, the precaution was taken of spreading the collection of data from the egg transfers and from the two kinds of control cross over the same period of time. So that the control animals should be strictly comparable with the experimental animals, with respect not only to time but also to other variables, the females chosen to provide the two kinds of control offspring were taken from stock cages of intended donors and recipients respectively.



TEXT-FIG. 1. The plan of the main experiment. Eggs of donor origin develop into mice with the agouti coat colour of the donor strain (C3H/Bi). These mice can therefore be distinguished from the step-father's offspring which are black pure-strain C57BL/How mice.

## EXPERIMENTAL RESULTS

The three kinds of mice (one experimental and two control types) were obtained in parallel over a period of 27 months. Altogether 197 transfers were made, 70 mice of donor origin were raised, and 67 were classified for vertebral type. The 70 mice of donor origin were obtained from the 51 recipients which raised litters. These had received an aggregate of 299 transferred eggs, so that for these mice the yield, 23·4 per cent., was similar to that reported elsewhere for this technique (McLaren & Michie, 1956*b*). However, when considered in relation to the total number of operations performed, the yield was very low, owing to the poor reproductive performance of the recipient C57BL/How females. This can be seen from Text-fig. 2, where a graphic record of the fate of the transfer operations is plotted month by month. A large proportion of the recipients failed to become pregnant. Of those which became pregnant a further fraction aborted, gave birth to dead litters, failed to nurse their litters, or failed entirely to give birth and had to be killed. The incidence of all these features was similar in control pregnancies of C57BL/How females, so they are not attributable to the experimental procedures.



TEXT-FIG. 2. The fate of the 197 transfer operations of the main experiment plotted month by month over the experimental period. The 27 litters containing mice of donor origin are indicated by the black squares.

Bearing in mind that only the female offspring can give rigorous discrimination between the cytoplasmic and uterine alternatives, owing to the possible complication of sex-linked genes with vertebral effects, we may now turn to Table 2, where the results relating to the experimental and control female offspring are summarized. The experimental mice appear unambiguously to resemble the control $F_1$ offspring of recipient-strain mothers and to differ from the control $F_1$ offspring of donor-strain mothers, thus showing that the effect is uterine. The corresponding data for male offspring are set out in Table 3. As far as they go they also suggest a host effect, but the relative predominance of the 5/5 type among males of all kinds diminishes the statistical information which can be extracted from them. Their failure to deviate as far as the reciprocal (C57BL ♀ × C3H ♂) controls might be due either to sex linkage or to random sampling.

TABLE 2

*Vertebral types of (C3H ♀ × C57BL ♂) $F_1$ female progeny reared in the uteri of C57BL females, compared with females of the two reciprocal $F_1$ control groups*

| Type of cross | Vertebral type of $F_1$ ♀♀ | | | | | 5/5 as per cent. of total |
|---|---|---|---|---|---|---|
| | 5/5 | 6/5 | 5/6 | 6/6 | Total | |
| (C3H ♀ × C57BL ♂) in C57BL uteri. | 7 | 3 | 1 | 19 | 30 | 23·3 |
| Control C3H ♀ × C57BL ♂ . . | 30 | 10 | 1 | 8 | 49 | 61·2 |
| Control C57BL ♀ × C3H ♂ . . | 16 | 10 | 1 | 34 | 61 | 26·2 |

TABLE 3

*Vertebral types of (C3H ♀ × C57BL ♂) $F_1$ male progeny reared in the uteri of C57BL females, compared with males of the two reciprocal $F_1$ control groups*

| Type of cross | Vertebral type of $F_1$ ♂♂ | | | | | 5/5 as per cent. of total |
|---|---|---|---|---|---|---|
| | 5/5 | 6/5 | 5/6 | 6/6 | Total | |
| (C3H ♀ × C57BL ♂) in C57BL uteri. | 24 | 7 | 2 | 4 | 37 | 64·9 |
| Control C3H ♀ × C57BL ♂ . . | 24 | 5 | 0 | 0 | 29 | 82·8 |
| Control C57BL ♀ × C3H ♂ . . | 34 | 8 | 6 | 16 | 64 | 53·1 |

Returning therefore to the data on females, we must apply a test of statistical significance which is impervious to the phenomena of between-sides correlation and litter-mate correlation which are characteristic features of vertebral variation in mice. An appropriate procedure is to calculate for each litter the mean number of unsacralized lumbar transverse processes per mouse: for an individual mouse this number can take the values 10, 11, or 12, corresponding to the 5/5, asymmetrical, and 6/6 phenotypes respectively. An analysis of variance is then performed on the litter means so obtained, weighting each mean by the number of mice contributing to it. A purely empirical estimate of sampling error is thus used, requiring no *a priori* assumptions (unlike the use of a standard $\chi^2$ or other binomially based test) concerning the distribution of sacralization between sides or among litters. The analysis is set out in Table 4, from which it appears that the results conflict with the cytoplasmic hypothesis at the 0·001 probability level, but are in excellent agreement with the hypothesis that the difference between the control reciprocal $F_1$ females is attributable to the uterine environment.

It remains to satisfy ourselves that this result is not an artefact arising from imperfect temporal matching between experimental and control litters. In

### TABLE 4

*Analysis of variance of the experimental results (females only)*

Significant mean square ratios for the first or second comparison would support the cytoplasmic or uterine hypothesis respectively.

| | *Sums of squares* | *Degrees of freedom* | *Mean square* | |
|---|---|---|---|---|
| 1st comparison: (C3H ♀ × C57BL ♂) in C57BL uteri compared with control C57BL ♀ × C3H ♂ | | | | Mean square ratio = |
| Between groups . . . . . . | 0·2214 | 1 | 0·2214 | 0·27 |
| Within groups . . . . . . | 35·1785 | 43 | 0·8181 | $P > 0{\cdot}5$ |
| TOTAL . . . . . . . | 35·3999 | 44 | | |
| 2nd comparison: (C3H ♀ × C57BL ♂) in C57BL uteri compared with control C3H ♀ × C57BL ♂ | | | | Mean square ratio = |
| Between groups . . . . . . | 13·4118 | 1 | 13·4118 | 16·06 |
| Within groups . . . . . . | 34·2224 | 41 | 0·8347 | $P < 0{\cdot}001$ |
| TOTAL . . . . . . . | 47·6342 | 42 | | |

### TABLE 5

*Vertebral types of experimental and control female progeny, divided chronologically into four groups*

| | Control C3H ♀ × C57BL ♂ | | (C3H ♀ × C57BL ♂) *in* C57BL *uteri* | | Control C57BL ♀ × C3H ♂ | |
|---|---|---|---|---|---|---|
| *Date of birth of mice* | *5/5* | *Other* | *5/5* | *Other* | *5/5* | *Other* |
| Mar.–Sept. 1955 . . | 4 | 6 | 3 | 5 | 4 | 8 |
| Oct.–Dec. 1955 . . | 8 | 5 | 2 | 6 | 4 | 7 |
| Mar.–Dec. 1956 . . | 9 | 2 | 1 | 5 | 2 | 13 |
| Jan.–Aug. 1957 . . | 9 | 6 | 1 | 7 | 6 | 17 |
| TOTAL . . . . | 30 | 19 | 7 | 23 | 16 | 45 |

### TABLE 6

*Comparison of three groups of C57BL/How ♀ × C3H/Bi ♂ $F_1$ hybrid mice*

The data for the first group, born during 1953–4, are taken from McLaren & Michie (1956*a*). The discrepancy in the sex of one mouse is due to an error in our previous tabulation. The data for the second group, born during 1955–7, are from Tables 2 and 3 above. The data for the third group, born in 1958, are from Table 7 below.

| | | *Vertebral types* | | | | | |
|---|---|---|---|---|---|---|---|
| *Group* | *Sex* | *5/5* | *6/5* | *5/6* | *6/6* | *Total* | *6/6 as per cent. of total* |
| 1 | ♀♀ | 7 | 4 | 3 | 48 | 62 | 77·4 |
| | ♂♂ | 15 | 8 | 2 | 34 | 59 | 57·6 |
| 2 | ♀♀ | 16 | 10 | 1 | 34 | 61 | 55·7 |
| | ♂♂ | 34 | 8 | 6 | 16 | 64 | 25·0 |
| 3 | ♀♀ | 7 | 8 | 5 | 42 | 62 | 67·7 |
| | ♂♂ | 19 | 7 | 2 | 26 | 54 | 48·1 |

Table 5 the data are divided into four successive periods of time. Although non-significant fluctuations within each of the three series occur from one period to another, it can be seen that they do not in themselves contribute to the overall effect, since the experimental mice consistently follow the host-type rather than the donor-type controls, except in the first period in which it happens that the two types of control females differ little from each other.

At the same time we have striking evidence that the policy of raising matched 'contemporaneous' controls was prudent and necessary. A comparison is made in Table 6 between data collected from three groups of C57BL/How ♀ × C3H/Bi ♂ $F_1$ mice during three successive periods of time. The proportion of 6/6 mice was high during the first period, fell to a relatively low level during the second, and rose again during the last period. The differences are substantial and statistically highly significant. They may be due to genetic drift of the type which we have previously reported (McLaren & Michie, 1955) or to undetected changes in the environment or in husbandry procedures. Whatever the cause, this result again underlines the necessity of carefully matching the control and experimental animals in work of this kind.

### *Do embryos of different genetic constitution affect one another?*

Our recipient C57BL/How females were mated to C57BL/How males, and therefore simultaneously carried embryos derived from native (inbred) and transferred ($F_1$ hybrid) eggs. Twenty-four native progeny from mixed litters were classified for vertebral type: all proved to be of the 6/6 type characteristic of the C57BL/How strain. Further, the vertebral type of the young derived from transferred eggs was in no way related to the number of native young in the same litter. We therefore conclude that as far as the number of lumbar vertebrae is concerned, there is no interaction between embryos of different genetic constitution gestated in the same uterus.

### *Is the effect of egg transfer host-specific?*

It might be argued that the effect which we have observed is due to some hypothetical tendency for egg transfer as such to raise the number of lumbar vertebrae in the fostered embryos, and is unrelated to the recipient strain used.

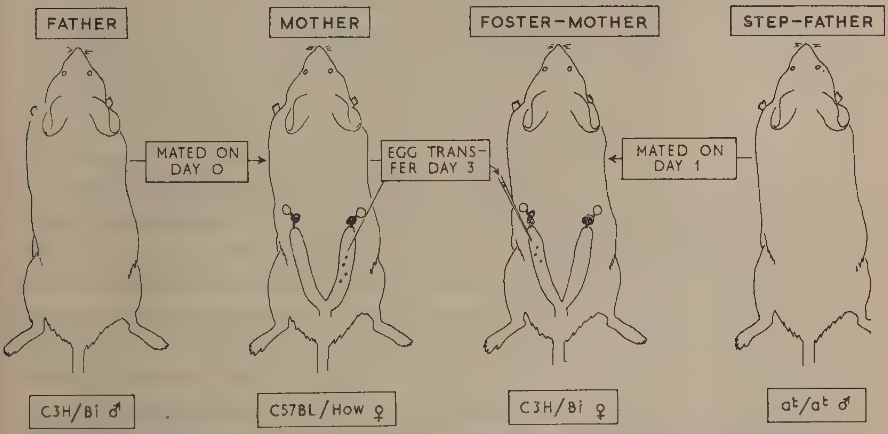
In order to obtain an experimental answer to this objection, we have performed a number of egg transfers in the reverse direction, as illustrated in Text-fig. 3. 'Contemporaneous' controls of both types were again raised.

On the 'non-specific' hypothesis we should expect the experimental mice to deviate strongly in the direction of 6 lumbar vertebrae, even more than the donor-type (mainly 6/6) controls. It is clear from the results summarized in Table 7 that they do not do so, but resemble the recipient-type controls, as would be expected if the effect of transfer were due to the specific nature of the uterine environment provided by the recipient.

TABLE 7

*Vertebral types of (C57BL ♀ × C3H ♂) $F_1$ progeny reared in the uteri of C3H females, compared with mice of the two reciprocal $F_1$ control groups*

| Sex | Type of cross | Vertebral type of $F_1$ progeny | | | | | 5/5 as per cent. of total |
|---|---|---|---|---|---|---|---|
| | | 5/5 | 6/5 | 5/6 | 6/6 | Total | |
| Females | (C57BL ♀ × C3H ♂) in C3H uteri. | 4 | 1 | 0 | 0 | 5 | 80·0 |
| | Control C3H ♀ × C57BL ♂ . . | 17 | 1 | 1 | 3 | 22 | 77·3 |
| | Control C57BL ♀ × C3H ♂ . . | 7 | 8 | 5 | 42 | 62 | 11·3 |
| Males | (C57BL ♀ × C3H ♂) in C3H uteri. | 4 | 1 | 0 | 0 | 5 | 80·0 |
| | Control C3H ♀ × C57BL ♂ . . | 10 | 0 | 0 | 0 | 10 | 100·0 |
| | Control C57BL ♀ × C3H ♂ . | 19 | 7 | 2 | 26 | 54 | 35·2 |



TEXT-FIG. 3. The plan of the 'reverse transfer' experiment which was done to test the specificity of the uterine effect. In this case the mice of native origin can be distinguished from those of donor origin by their light belly colour inherited from the black-and-tan ($\mathbf{a}^t$) male.

## DISCUSSION

The results which we have described indicate that the maternal effect upon vertebral type in mice is due to the specific influence of the uterine environment.

As mentioned earlier, the uterine environment has been shown in rabbits and in sheep to influence the weight of the young at birth. More recently Brumby (personal communication) has found the same in mice. It is therefore of interest to inquire whether vertebral type is in any way associated with birth-weight, as are some other skeletal characters. Deol & Truslove (1957), studying sacralization of the 26th vertebra in the C57BL/Gr strain of mice, found no significant association with birth-weight in extensive control material. Dietary deficiencies, however, depressed both birth-weight and the already low incidence of sacralization; and there is an indication that the animals in which sacralization occurred were those whose birth-weights were least affected by the deficient diet.

In our own material, some of the mice in the experimental and control series were weighed on the day of birth. The relevant data are summarized in Table 8, and although the number of observations is small, they suffice to show that there is no marked association of number of lumbar vertebrae with birth-weight. This is not surprising, since in the earlier work (McLaren & Michie, 1954, 1955) we found vertebral type to be independent of the number of young in the litter, a variable on which birth-weight is profoundly dependent. This independence was again confirmed in the present material, and is in agreement with Green & Green's (1946) finding that in the Bagg albino strain, sacralization of the 26th vertebra is not related to the number of foetuses in the uterine horn. We therefore feel justified in concluding that in our material vertebral type is independent not only of the overall prenatal growth rate, but also of any other factor of the prenatal environment which is affected by the number of embryos in the uterus.

TABLE 8

*Birth-weight in two groups of mice, classified according to vertebral type*

| | | *Vertebral type* | | |
|---|---|---|---|---|
| *Type of cross* | | *5/5* | *6/5 or 5/6* | *6/6* |
| (C3H ♀×C57BL ♂) in C57BL uteri | Average weight at birth | 1·20<br>±0·115 | 1·33<br>±0·137 | 1·30<br>±0·075 |
| | No. of mice weighed | 8 | 4 | 11 |
| Control C57BL ♀×C3H ♂ . . | Average weight at birth | 1·22<br>±0·085 | 1·12<br>±0·075 | 1·19<br>±0·054 |
| | No. of mice weighed | 15 | 6 | 18 |

On the other hand, the number of lumbar vertebrae might be critically affected by the rate of embryonic growth before and during the early stages of prenatal life at which sacralization is known to be determined (days $7\frac{1}{2}$–$9\frac{1}{2}$), since at this stage the embryonic demands on nourishment and space are still relatively small, and growth is therefore unlikely to be dependent on the number of embryos. For the same reason it seems unlikely that at this stage, limits to growth should be set by the sheer quantities of nourishment or space provided by the mother. One would rather look for the deciding factor in the concentration in the maternal blood, or rate of transport to the embryo, of some particular substance or substances with specific effect upon the critical developmental stage. It is perhaps along these lines that an explanation should be sought for the finding of Deol & Truslove (1957), mentioned earlier, that C57BL mice maintained on deficient diets showed a lower incidence of sacralization of vertebra 26 than control mice of the same strain.

Uncertainties of interpretation also attend the important demonstration by

Russell & Russell (1950) that the frequency of sacralization of vertebra 26 is decreased by X-irradiation on days $7\frac{1}{2}$–$9\frac{1}{2}$. Since the pregnant females were not shielded during the irradiation of their embryos it is not clear whether the X-rays acted directly upon the embryos or indirectly by affecting the mothers. Even if the mothers were shielded a positive result of such an experiment would not be entirely unambiguous, since damage to the uterine vascular system or to the placentae would not be excluded. Lengerova (1957) finds evidence that X-irradiation of the gravid uteri of shielded pregnant rats causes partial breakdown of the placental barrier.

### *Practical implication of work on uterine effects*

Since man is a mammal the role of the uterine environment, particularly in the production of abnormality, is an inevitable focus of clinical interest. Pathological states known to be under the influence of the maternal-foetal relationship include mongolism, haemolytic disease of the newborn, cleft palate, and polydactyly, and many others are suspected of belonging to the same category. In addition a number of human skeletal variants of lesser clinical importance are known to depend on variables of the maternal physiology, some of them socially conditioned.

In agriculture there is the prospect that in the rather distant future egg transfer may become practicable on a commercial scale. Outstanding females could then be propagated, or their offspring given cheap inter-continental transport, by transferring large numbers of their fertilized eggs into batteries of genetically inferior recipients. It will then be of acute importance to know whether the offspring of such transfers will remain entirely faithful to the donor's type, or whether they may acquire some of the less desirable qualities of the recipient type. A very partial answer to this question can be given on the basis of the experimental work which we have described, namely, that the possibility of such host effects has been established.

In the light of the results with vertebral variation we must be prepared to extend our picture of the genetic control of morphogenetic processes and to envisage their regulation not only by the action of the embryo's own genes but also by the action of the genes of the maternal organism in which the embryo is gestated.

## SUMMARY

1. C3H/Bi and C57BL/How inbred strains of mice are known to differ from one another in number of lumbar vertebrae. Reciprocal $F_1$ hybrids between the two strains tend to resemble the maternal strain in this respect. Since the difference between reciprocal hybrids is shown in females as well as in males, it cannot be attributed to sex linkage.

2. Analysis by means of egg transfer has shown that this reciprocal difference

in the number of lumbar vertebrae is due to an influence exerted by the mother through the uterine environment, rather than through the cytoplasm of the egg.

3. The number of lumbar vertebrae is not affected by the operation of egg transfer in itself, but only by the specific influence of the host uterine environment.

4. In our material vertebral type is not influenced by the genetic constitution of the other embryos in the uterus, nor by their number. There is no marked dependence of number of lumbar vertebrae upon birth-weight.

5. We conclude that in this instance the uterine environment does not act through promotion or inhibition of overall embryonic growth, but exerts a specific morphogenetic influence upon the unborn young.

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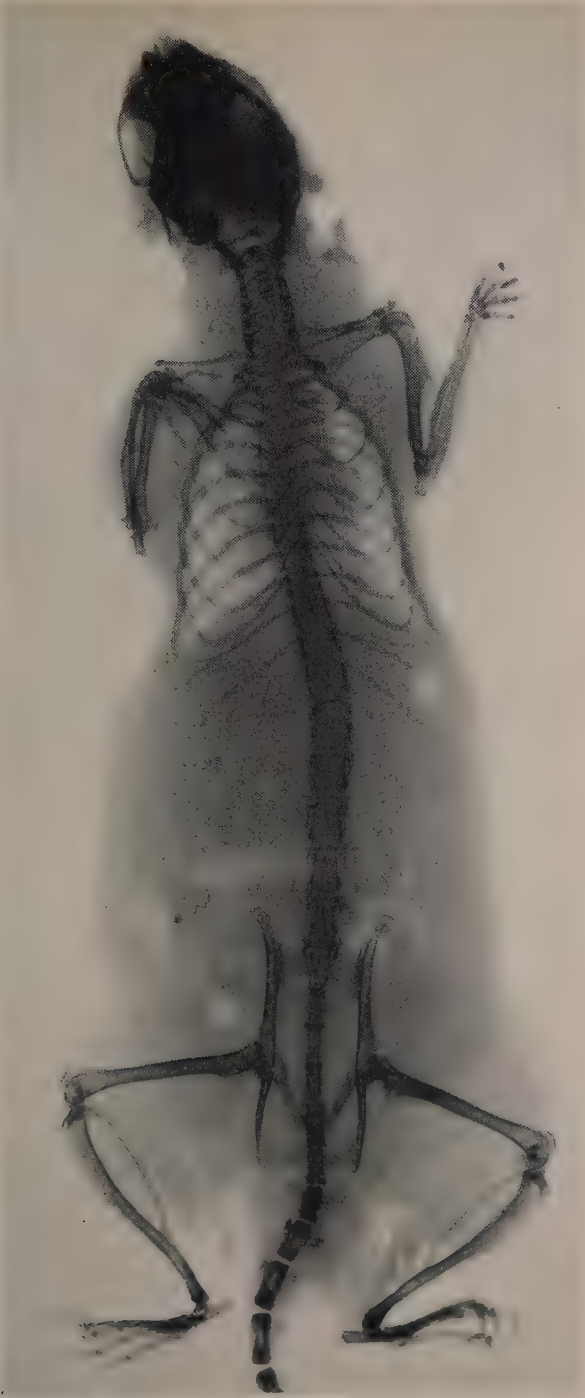

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## EXPLANATION OF PLATE

X-ray photograph of a mouse with 5 lumbar vertebrae. × 2.

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